PHARMACOGNOSTICAL, PHYTOCHEMICAL AND MEMORY STRENGTHENING ACTIVITY ON THE SEEDS OF *Abelmoschus moschatus* Medic.

A Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
CHENNAI – 600032

In partial fulfillment of the requirements for the award of degree of

MASTER OF PHARMACY IN PHARMACOGNOSY

Submitted by

Reg No.261220656

Under the guidance of

Dr.R.VADIVU M.Pharm., Ph.D.,

Department of Pharmacognosy
College of Pharmacy
MADRAS MEDICAL COLLEGE
Chennai – 600003
April - 2014

Dr .A. JERAD SURESH, M.Pharm., Ph.D.,
Principal,
College of Pharmacy,
Madras Medical College,
Chennai - 600003.

CERTIFICATE

This is to certify that this dissertation work entitled "**PHARMACOGNOSTICAL, PHYTOCHEMICAL AND MEMORY STRENGTHENING ACTIVITY ON THE SEEDS OF *Abelmoschus moschatus* Medic.,**" submitted by the candidate bearing the **Reg. No. 261220656**, final year M.Pharm (Pharmacognosy) student, in partial fulfillment of the requirements for the award of degree in **Master of Pharmacy in Pharmacognosy** by **The Tamil Nadu Dr.M.G.R.Medical University, Chennai-600032**, is a bonafide record of work done by her in the Department of Pharmacognosy, Madras Medical College, Chennai, during the academic year 2013 – 2014 under the guidance of **Dr. R. VADIVU, M.Pharm.,Ph.D.,** Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai – 600003.

Dr. A. JERAD SURESH, M.Pharm, Ph.D.,

Place: Chennai – 03

Date:

Dr .N. Jayshree, M.Pharm., Ph.D.,
Professor and Head,
Department of Pharmacognosy,
College of Pharmacy,
Madras Medical College,
Chennai- 600003

CERTIFICATE

This is to certify that this dissertation work entitled "**PHARMACOGNOSTICAL, PHYTOCHEMICAL AND MEMORY STRENGTHENING ACTIVITY ON THE SEEDS OF *Abelmoschus moschatus* Medic.,**" submitted by the candidate bearing the **Reg. No. 261220656**, final year M.Pharm (Pharmacognosy) student, in partial fulfillment of the requirements for the award of degree in **Master of Pharmacy in Pharmacognosy** by **The Tamil Nadu Dr.M.G.R.Medical University, Chennai-600032**, is a bonafide record of work done by her in the Department of Pharmacognosy, Madras Medical College, Chennai, during the academic year 2013 – 2014 under the guidance of **Dr. R. VADIVU, M.Pharm.,Ph.D.,** Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai – 600003.

Dr. N. JAYSHREE, M.Pharm, Ph.D.,

Place: Chennai – 03

Date:

Dr. R.Vadivu , M.Pharm., Ph.D.,
Tutor,
Department of Pharmacognosy,
College of Pharmacy,
Madras Medical College,
Chennai- 600003

CERTIFICATE

This is to certify that this dissertation work entitled "**PHARMACOGNOSTICAL, PHYTOCHEMICAL AND MEMORY STRENGTHENING ACTIVITY ON THE SEEDS OF *Abelmoschus moschatus* Medic.,**" submitted by the candidate bearing the **Reg. No. 261220656**, final year M.Pharm (Pharmacognosy) student, in partial fulfillment of the requirements for the award of degree in **Master of Pharmacy in Pharmacognosy** by **The Tamil Nadu Dr.M.G.R.Medical University, Chennai-600032**, is a bonafide record of work done by her in the Department of Pharmacognosy, Madras Medical College, Chennai, during the academic year 2013 – 2014 under the guidance of **Dr. R. VADIVU, M.Pharm.,Ph.D.,** Department of Pharmacognosy, College of Pharmacy, Madras Medical College, Chennai – 600003.

Dr. R.VADIVU, M.Pharm., Ph.D

Place: Chennai – 03

Date:

ACKNOWLEDGEMENT

The study was carried out at the Department Of Pharmacognosy, Madras Medical College, Chennai, during the year 2012-2013. With immense gratitude I acknowledge the following persons for their pivotal role in completion of the study.

I whole heartedly express my high esteem and deep sense of gratitude to the respectable Dean **Dr .V. Kanagasabai., M.D.,** Madras Medical College, Chennai, for providing me all facilities and support during the period of academic course work.

It is with the great pleasure, I record my thanks and gratitude to our Principal **Dr. A. Jerad Suresh., M.Pharm., Ph.D.,** College of Pharmacy, Madras Medical College, for providing the lab and administrative facilities to carry out the project work.

I express my deep sense of gratitude to **Dr. N. Jayshree, M.Pharm., Ph.D.,** Professor and Head, Department of Pharmacognosy, College of Pharmacy, Madras Medical College, for her support in completion of the project work.

I like to thank my guide **Dr. R. Vadivu, M.Pharm, Ph.D.,** for her invaluable support and guidance throughout the project. I am greatly indebted to her for the excellent advice, patience, moral support and help. Her right suggestions at right time have rescued me from various red tape crisis. Without her support, the project would not have been materialized and completed.

It's a great pleasure for me to acknowledge my sincere thanks to all the staff members **Dr. Radha, M.Pharm., Ph.D., Dr. P. Muthusamy M.Pharm., Ph.D., B.L., and Dr. R. Vijaya Bharathi, M.Pharm., Ph.D.,** of the Department of Pharmacognosy for their valuable support and excellent co-operation when needed.

I acknowledge my sincere thanks to **Dr. Joseph Dixon, B.V.Sc.,** special veterinary officer and **Mr. Kandaswamy,** Assistant of Animal Experimental House, Madras Medical College, Chennai-03 for providing the animals to carryout Pharmacological studies.

I wish to show my gratitude to **Mr.Chelladurai** for his efforts in collection of plant material.

A special word of thanks goes to the non-teaching staff members **Mrs. T.S. Lakshmi and Mrs. M. Kumudhavalli,** Department of Pharmacognosy, College of Pharmacy, MMC, Chennai-03.

I have no words to express my joy in thanking the Almighty for giving me the fabulous group of friends in **PG** and all other who are behind me supporting my endeavours.

I also extend my thanks to all those who have directly or indirectly helped me during this tenure.

I wish to thank **my father**, who taught me perseverance in life and **my mother** who was the reason behind all my success. I would like to thank **my sisters** and **brother** for sharing all my happiness and challenges throughout the course period.

The financial support provided by the **Tamilnadu Government** is gratefully acknowledged.

CONTENTS

S.No	Title	Page No
1	INTRODUCTION	1
2	REVIEW OF LITERATURE	11
3	AIM AND OBJECTIVE OF THE STUDY	13
4	RATIONALE FOR SELECTION	14
5	ETHNOBOTANICAL SURVEY	15
6	PLAN OF WORK	19
7	METHODOLODY 7.1 Pharmacognostical studies 7.2 Phytochemical studies 7.3 Selection of active extract 7.4 Pharmacological studies	 20 30 37 39
8	RESULTS AND DISCUSSION	46
9	SUMMARY AND CONCLUSION	81
10	REFERENCES	-

LIST OF TABLES

Table No	Title	Page No
1.	Herbals used for treatment of memory disorders	10
2.	Physico chemical constants	54
3.	Qualitative estimation of inorganic elements	55
4.	Quantitative estimation of inorganic elements	56
5.	Quantitative estimation of Heavy metals	56
6.	Percentage yield of successive extracts	59
7.	Qualitative Phytochemical analysis	60
8.	Behaviour of drug powder with different chemical reagents	61
9.	Total flavanoid content of ethyl acetate and ethanolic extracts	62
10.	Total phenolic content of ethyl acetate and ethanolic extracts	63
11.	Quantitative estimation of phytoconstituents	64
12.	Fluorescence analysis of powder	65
13.	Fluorescence analysis of extracts	66
14.	TLC profile of extracts	67
15.	Solvent system for HPTLC	68
16.	HPTLC profile of extracts	69
17.	In-vitro reducing power assay	72
18.	In-vitro hydrogen peroxide scavenging assay	73
19.	Effect on Transfer Latency	75
20.	Brain Acetyl cholinesterase level	76
21.	Brain Malondialdehyde Level	78
22.	Brain Reduced glutathione level	79

LIST OF FIGURES

Figure No	Title of figure	Page No
1.	Anatomy of brain	5
2.	Three parts, Three pounds	6
3.	The neuron forest and cell signaling	7
4.	Alzheimer's changes the whole brain	8
5.	Whole plant of *Abelmoschus moschatus*	14
6.	Seeds of *Abelmoschus moschatus*	14
7.	OECD guidelines	41
8.	Elevated plus maze model	43
9.	Macroscopy of seeds	46
10.	T.S of seed	47
11.	Palisade like Scleride layer	48
12.	Inner zone of seed	48
13.	Inner zone of testa	49
14.	Endosperm layer	49
15.	Middle zone of seed	50
16.	Embryo with folded cotyledons	50
17.	Single layered endosperm cells	51
18.	Outer zone contain polygonal epidermal cells	51
19.	Sclerenchyma cells	52
20.	Mucilage cells	52
21.	Testa	53
22.	Parenchyma and sclerenchyma cells	53
23.	Total flavanoid content	62
24.	Total phenolic content	63
25.	TLC profile for ethyl acetate extract	67
26.	TLC profile for ethanolic extract	67

27.	TLC profile for ethanolic extract.	68
28.	HPTLC profile of ethanolic extract	69
29.	In vitro reducing power assay	72
30.	In vitro hydrogen peroxide assay	73
31.	Transfer latency on 7^{th} and 8^{th} day	75
32.	AchE inhibitory activity	76
33.	Brain Malondialdehyde level	78
34.	Brain reduced glutathione level	79

Memory strengthening activity on seeds of *Abelmoschus moschatus*

S.Nandhini, R.Vadivu*, N.Jayshree

Department of Pharmacognosy

College Of Pharmacy

Madras Medical College

Chennai, Tamilnadu, India.

ABSTRACT

Alzheimer's disease is a progressive neurodegenerative disorder characterized by gradual decline in memory. The present study was undertaken to investigate the memory strengthening effect of *Abelmoschus moschatus Medic*. Ethanolic extract of seeds (100,200mg/kg. p.o) was administered for 7 successive days to young mice. Exteroceptive behavioral model such as elevated plus maze was employed to evaluate learning and memory. To delineate the mechanism by which AM exerts memory strengthening activity, the effect of AM on whole brain AchE, Brain Malondialdehyde content , Reduced Glutathione were also assessed. Piracetam (200mg/kg, i.p) was used as a standard drug. Pretreatment with AM (200,400mg, p.o) for seven successive days significantly improved learning and memory in mice and reversed the amnesia induced by diazepam (1mg/kg, i.p). AM also decreased whole brain AchE and Malondialdehyde content and increase the brain reduced glutathione. Hence *Abelmoschus moschatus Medic.* appears to be a promising candidate for improving memory, Anti cholinesterase activity and Anti oxidant property and it would be worthwhile to explore the potential of this plant in the management of dementia and Alzheimer's disease.

Key words: *Abelmoschus moschatus*, Alzheimer's disease, elevated plus maze model, Anti cholinergic.

1. INTRODUCTION

Memory is the ability of an individual to record sensory stimuli, events, information etc., retain them over short or long periods of time and recall the same at a later date when needed. Poor memory, lower retention and slow recall are common problems in today's stressful and competitive world. Age, stress and emotions are conditions that may lead to memory loss [(1)]

It is the process in which information is encoded, stored and retrieved. Encoding allows information that is from the outside world to reach our senses in the forms of chemical and physical stimuli. In this first stage we must change the information so that we may put the memory into the encoding process. Storage is the second memory stage or process. This entails that we maintain information over periods of time. Finally third process is the retrieval of information that we have stored. We must locate it and return it to our consciousness.

Human body is interconnected and is designed to perform many activities at the same time. Human memory is very crucial in predicting the daily routine performance.

There are many factors that determine the performance of human memory. A set of diseases affects the performance of the human brain are called as the memory related diseases or neurodegenerative diseases [(2)], One of the diseases is the Alzheimer's disease (AD) and that is much like the dementia, is characterized by the development of senile plaques and neurofibrillary tangles, which are associated with neuronal loss affecting to a greater extent cholinergic neurons in brain.

The disease progresses with the passage of time and as a person get older the disease also matures. Around 35 million patients suffer from Alzheimer's disease all over the world and this number is increasing with each passing second [(3)].

An epidemiological study reveals that dementia is largely hidden problem, especially in rapidly developing and heavily populated regions such as India, China and America [(3)]. Dementia associated with Alzheimer's disease is the most common cause of memory impairment or cognitive disability in elderly people.

ALZHEIMER'S COMMON WARNING SIGNS [4, 5]

Alzheimer is a disease which affects our memory and thinking capabilities. In its initial stages it affects our memory, but in later this cause adverse effects on daily life routine. Beware of the disease and consider various symptoms to stop the problem. It has some specific and clear symptoms in its initial stages like,

- ✓ The first most common sign is the memory loss. You may forget dates and events and then recall them latter. This state of forgetting things may be an initial sign of the brain disease.

- ✓ People start facing a problem in solving matters and daily plans and they may not be able to handle numbers and checkbooks to figure out the calculation. The challenges they face may be a warning sign of the growing Alzheimer's.

- ✓ People start facing problems in handling their daily home tasks. They even find difficult in recording programs and memorizing rules of the games.

- ✓ In some middle stages people face this problem and the warning sign is the time and place loss. They cannot recognize where they are and how much time has been passed.

- ✓ This brain disease also affects chromatic vision. They will not be recognizing colour and visual picture due to the effect of brain.

- ✓ One warning sign of Alzheimer's is forgetting words and spellings. People may not continue their speech and forgets what to speak later on. Difficulty in speaking and making words with letters is also a sign of disease.

- ✓ Other symptoms of the disease include leaving social activities and gathering, facing difficulties in decision making and talking with other people.

CAUSES FOR MEMORY LOSS [6, 7]

- ✓ **Medications:** A number of prescription and over-the-counter medications can interfere with or cause loss of memory. Possible culprits include: antidepressants, antihistamines, anti-anxiety medications, muscle relaxants, tranquilizers, sleeping pills, and pain medications given after surgery.
- ✓ **Alcohol, tobacco or drug use:** Excessive alcohol use has long been recognized as a cause of memory loss. Smoking harms memory by reducing the amount of oxygen that goes to the brain. Studies have shown that people who smoke find it more difficult to put faces with names than do nonsmokers. Illicit drugs can change chemicals in the brain that can make it hard to recall memories.
- ✓ **Sleep deprivation:** Both quantity and quality of sleep are important to memory. Getting too little sleep or waking frequently in the night can lead to fatigue, which interferes with the ability to consolidate and retrieve information.
- ✓ **Depression and stress:** Being depressed can make it difficult to pay attention and focus, which can affect memory. Stress and anxiety can also get in the way of concentration. When you are tense and your mind is over stimulated or distracted, your ability to remember can suffer. Stress caused by an emotional trauma can also lead to memory loss.
- ✓ **Nutritional deficiency:** Good nutrition, including high-quality proteins and fats are important for proper brain function. Deficiencies in vitamin B_1 and B_{12} specifically can affect memory.
- ✓ **Head injury:** A severe hit to the head, from a fall or automobile accident, can injure the brain and cause both short- and long-term memory loss. Memory may gradually improve over time.
- ✓ **Stroke:** A stroke occurs when the blood supply to the brain is stopped due to the blockage of a blood vessel to the brain or leakage of a vessel into the brain. Strokes often cause short-term memory loss. A person who has had a stroke may have vivid memories of childhood events but be unable to recall what he or she had for lunch.
- ✓ **Dementia:** Dementia is the name for progressive loss of memory and other aspects of thinking that are severe enough to interfere with the ability to function in daily activities.

Although there are many causes of dementia, including blood vessel disease, drug or alcohol abuse, or other causes of damage to the brain, the most common and familiar is Alzheimer's disease. Alzheimer's disease is characterized by a progressive loss of brain cells and other irregularities of the brain.

- ✓ **Other causes:** Other possible causes of memory loss include an underactive or overactive thyroid gland and infections such as HIV, tuberculosis, and syphilis that affects the brain.

ANATOMY OF BRAIN [8]

Brain is the most complex of all organs. It hears, sees, feels, tastes, commands the limbs, talks, reads, writes, makes decisions and accomplishes numerous other functions. Most importantly for our interests, it is the organ of memory.

Our ears may hear the concert; our eyes may see the famous painting; our fingers may feel the silk nightgown; our nose may smell, our mouth may taste that delicious chocolate, but only our brain remembers what the experience was like, where it happened and the numerous other details involved. So, although the sensory abilities described above are not typically affected by Alzheimer's disease, the memory of these experiences and perhaps the appreciation of them can be markedly affected.

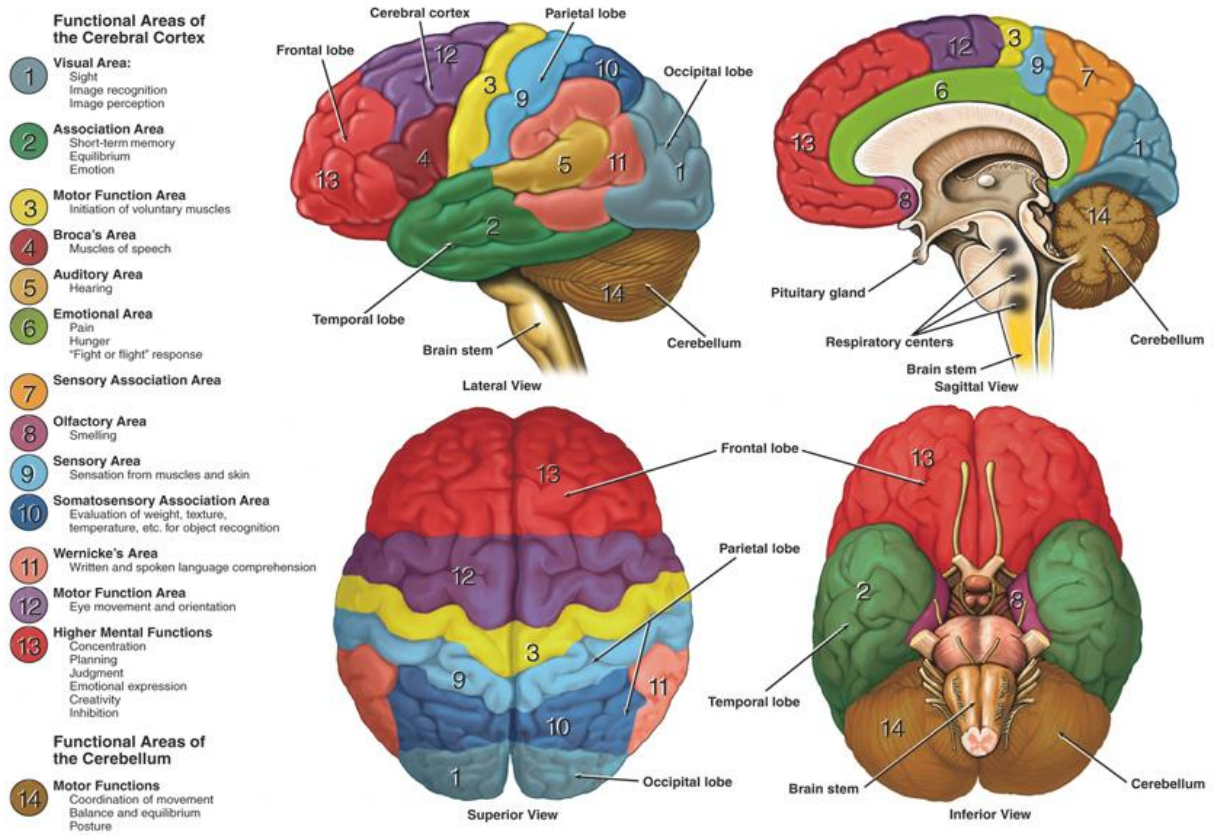


Figure 1 Anatomy of brain

Our brain is divided into right and left halves. The left half controls movement on the body's right side, the right half controls the body's left side.

THREE PARTS, THREE POUNDS

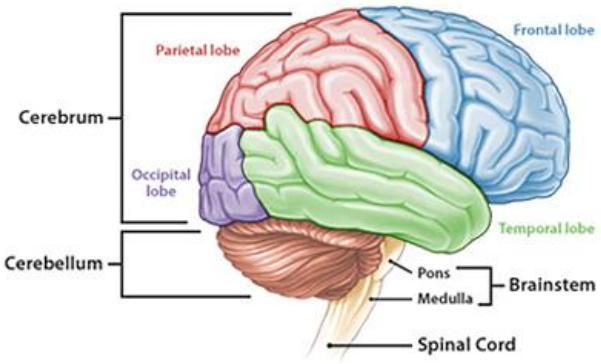


Figure 2.Parts of the brain

Our brain is our most powerful organ, yet weighs only about three pounds. It has a texture similar to firm jelly.

It has three main parts

- ✓ The **cerebrum** fills up most of our skull. It is involved in remembering, problem solving and feeling. It also controls movement. The wrinkled surface is a specialized outer layer of the cerebrum called the cortex.

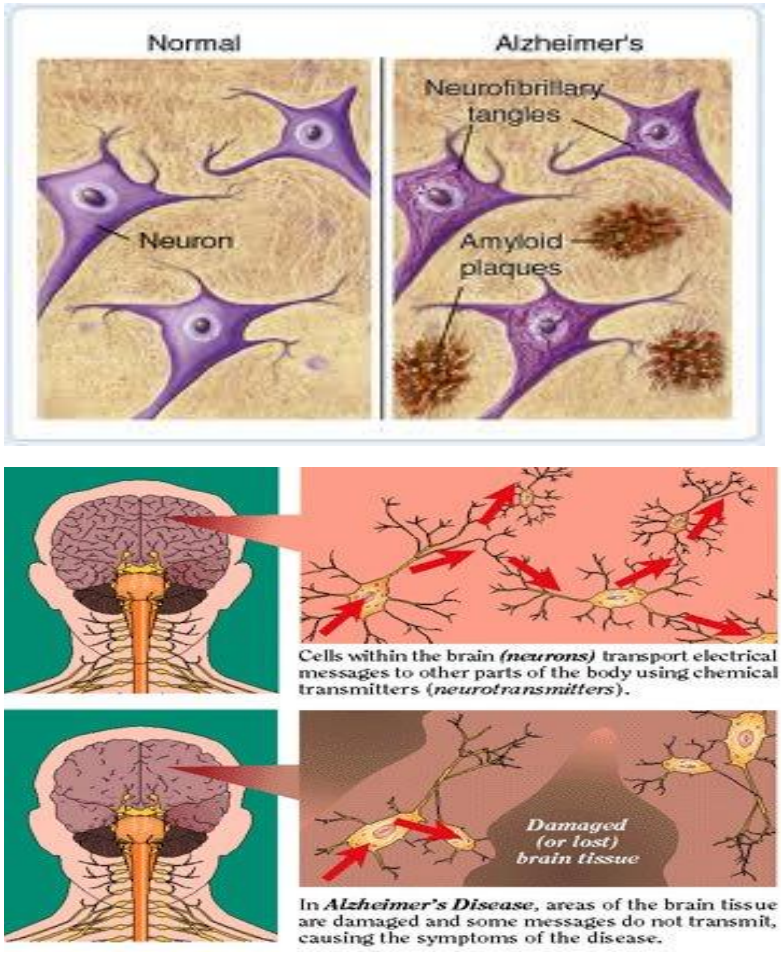
Specific regions of cortex:

1. Interpret sensations from our body and sights, sounds and smells from the outside world.
2. Generate thoughts, solve problems and make plans, form and store memories, control voluntary movement.

- ✓ The **cerebellum** sits at the back of our head, under the cerebrum. It controls coordination and balance.

- ✓ The **brain stem** sits beneath our cerebrum in front of our cerebellum. It connects the brain to the spinal cord and controls automatic functions such as breathing, digestion, heart rate and blood pressure.
- ✓ Our brain is nourished by one of our body's richest networks of blood vessels. With each heartbeat, arteries carry about 20 to 25% of our blood to our brain, where billions of cells use about 20% of the oxygen and fuel our blood carries.

THE NEURON FOREST AND CELL SIGNALING



Figures 3. The neuron forest and cell signaling

- ✓ An adult brain contains about 100 billion nerve cells or neurons with branches that connect at more than 100 trillion points are called as "neuron forest".
- ✓ Signals travelling through the neuron forest form the basis of memories, thoughts and feelings. Neurons are the chief type of cell destroyed by Alzheimer's disease.

- ✓ Signals that form memories and thoughts move through an individual nerve cell as a tiny electrical charge. Nerve cells connect to one another at synapses. When a charge reaches a synapse, it may trigger release of tiny bursts of chemicals called neurotransmitters. The neurotransmitters travel across the synapse; carrying signals to other cells, scientists have identified dozens of neurotransmitters.
- ✓ Alzheimer's disease disrupts both the way electrical charges travel within cells and the activity of neurotransmitters.

ALZHEIMER'S CHANGES THE WHOLE BRAIN

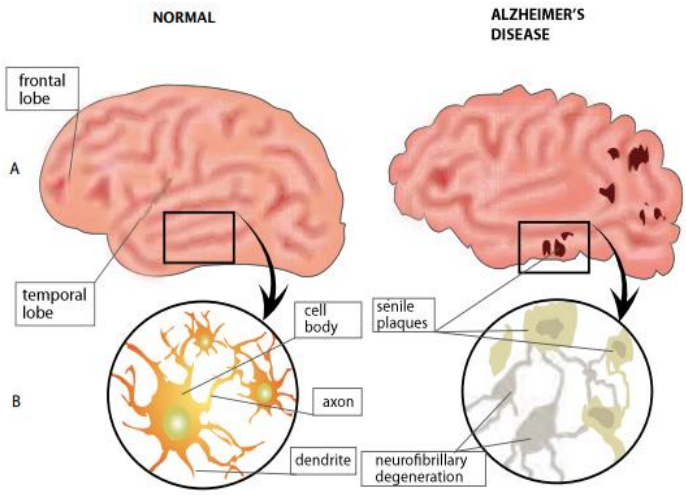


Figure 4.Alzheimer's changes the whole brain

- ✓ Alzheimer's disease leads to nerve cell death and tissue loss throughout the brain. Over time, the brain shrinks dramatically, affecting nearly all its functions.

Reducing oxidative stress by antioxidants, protecting brain inflammatory lesions using anti inflammatory drugs and facilitation of brain cholinergic neurotransmission with anti cholinesterase are some positive approach to management of AD[(4)]. Due to increase incidence of side effects of allopathic medicine (both nootropic and cholinesterase inhibitors) more research will be manifested towards the use of natural resources like medicinal plants for the management of various cognitive disorders.

The nature provides a new opportunity to regain one's full mental capacity. A number of herbs traditionally employed in the Indian system of medicine "Ayurveda" have yielded positive results.

HERBAL MEDICINE [9, 10]

Plants have been one of the important sources of medicine ever since the dawn of human civilization. In spite of tremendous developments in the field of allopathy, herbs are staging a comeback and "Herbal Renaissance" is happening all over the globe.

Even as we enter into the new century with its existing prospect of gene therapy, herbal medicines remain one of the common forms of therapy available to the world population. The acceptance and recognition of the herbal medicine has been in part due to the acknowledgement of the value of traditional medicine in Indigenous Pharmacopoeias, the incorporation of some medicines derived from these sources into Pharmaceuticals, the need to make health care affordable to all and the perception that pharmaceutical drugs are expensive and have more side effects. Another important perception fomenting this interest is that natural remedies are somehow safer and more efficacious than remedies that are pharmaceutically derived.

IMPORTANCE OF HERBAL MEDICINE

According to WHO (2001) 60% of the world's population depend on traditional medicine and 80% of the population in developing countries depend almost entirely on traditional medical practices, in particular herbal medicine for their primary healthcare needs.

As for developed countries, it is reported that sufferers of chronic diseases are turning to herbal remedies as alternatives to modern synthetic drugs. This renewed interest in the use of herbal medicine is to be motivated by several factors such as side effects of modern drugs, the effectiveness of plant remedies and high cost of synthetic drugs. The use of and search for drugs and dietary supplements derived from plants have accelerated in recent years.

Table 1.Herbals used for treatment of memory disorders [11-16]

S.No	Biological name	Synonym	Active constituents
1.	*Baccopa monnieri*	Brahmi	Triterpene glycoside
2.	*Valriana wallichi*	Tagara	Flavones glycoside
3.	*Nardostachys jatamansi*	Jatamansi	Flavonoids
4.	*Withania somnifera*	Ashwaganda	Withenolides
5.	*Terminalia arjuna*	Arjuna	Alkaloids
6.	*Piper longum*	Pippali moola	Alkaloids
7.	*Myristica fragrance*	Jatiphala	Volatile oil
8.	*Acorous calamus*	Vacha	Phenolic compounds
9.	*Rauwolfia serpentine*	Sarpagandha	Alkaloids
10.	*Celastrus paniculatus*	Jyothismati	Volatile oil.

Drug used for Alzheimer's which contain isolated plant constituents [17-19]

Huperizine A is an alkaloid with a potent acetyl cholinesterase inhibitory activity isolated from the Chinese club moss *Huperzia serrata*. Huperizine A is currently available in the USA as "Nutraceuticals" or "functional *food*".

Galantamine is an alkaloid used for symptomatic treatment of patients with early onset Alzheimer's disease. It was initially isolated from the snowdrop (*Galanthus woronowii*) in the early 1950's and has since been found in other plants in the family Amaryllidaceae. Galantamine slows the process of neurological degeneration by inhibiting AchE. Due to the limited availability of the plants of origin of this compound, Galantamine is now produced by total synthesis.

Considering the importance of herbal medicine in memory strengthening activity, the present study was aimed to investigate the effects of *Abelmoschus moschatus* plant on memory strengthening and brain cholinesterase activity in mice.

2. REVIEW OF LITERATURE

Pharmacognostical Review:

1. Ilaria Molfetta, Lucia Ceccarini, Mario Macchia, Guido Flamini, Pierluigi Cioni (2013) investigated ***Abelmoschus esculentus*** and Abelmoschus moschatus seeds production and analysis of the volatile compounds.[22]
2. Dubey Kumara Priyanka and Datta K.Animesh (2012) investigated induced Mutagenesis in ***Abelmoschus moschatus*** **Medic**.[23]
3. Asish R. Warghat, Nandkishor H. Ram Pure and Prashant Wagh, (2011) evaluated the *in vitro* callus induction of ***Abelmoschus moschatus*** **Medic.,** by using different hormone concentration and they reported that the good callus induction was found in MS medium supplemented with 0.3mg/1BAP and 2mg/NAA from first leaf explants.[24]

Phytochemical review:

1. AJM. Christina, P.Muthumani (2012) reported the Phytochemical studies on leaves of ***Abelmoschus moschatus*** **Medic.**[25]

Pharmacological review:

1. AJM. Christina, P.Muthumani (2013) investigated the protective effect of the hydro alcoholic extract of ***Abelmoschus moschatus*** **Medic.,** against Ethylene glycol induced Urolithiasis and it's possible underlying Mechanism using Male Wistar Albino Rats.[25]
2. AJM.Christina, P.Muthumani (2012) reported the ***Abelmoschus moschatus*** whole plant exhibited significant diuretic activity as evidenced by increase in total urine volume and the urine concentration of sodium, potassium and chloride.[26]
3. Abishek Kumar Singh, Sanjiv Singh, H.S. Chandel (2012) Evaluated the Hepato protective activity of ***Abelmoschus moschatus*** seed extract against Paracetamol and Ethanol induced hepato toxicity.[27]
4. RU Li, Changdong wang, Tao Chen, Peng Chen (2012) reported quantitative proteomic analysis of cold-responsive proteins in ***Abelmoschus Moschatus***.[28]

5. Mir ZGul, Lapakshi M Bhakshu, Farhan ahmad, Anand K Kondapi (2011) evaluated that the seed and leaf extract of ***Abelmoschus moschatus Medic.***, possess significant Anti oxidant Activity, Moderate Anti Bacterial activity, Anti proliferative activity against two human cancer cell lines.[29]
6. Priti Maheshwari, Anil Kumar (2009) reported the Anti Microbial activity of ***Abelmoschus moschatus*** Leaf Extract.[30]
7. Liu IM, Tzeng IT, Liou SS, Lan TW (2007) evaluated the improvement of insulin sensitivity in obese Zucker rats by Myricetin extracted from ***Abelmoschus moschatus*** **Medic.,**[31]
8. D.Sahoo, K.S Jena, P K Rout and T.R.Rao (2003) investigated two stage solvent extraction of seeds of ***Hibiscus abelmoschus*** **Medic.**[32]
9. G.Ramu, G Krishna Mohan, KN Jayaveera, N Suresh, Chandra Prakash(2002) reported that Seed starch obtained from ***Abelmoschus moschatus*** was employed as a disintegrant to paracetamol tablets at concentration of 2.5-10.0%w/w.[33]
10. R.Pandey, A Kalra, N Katiyar, S Kumar (2001), Nematicidal activity in flowers of ***Abelmoschus moschatus*** and aromatic plants.[34]

3. AIM AND OBJECTIVE OF THE STUDY

Poor memory, lower retention and slow recall are common problems in today's stressful and competitive world. Age, stress and emotions are conditions that may lead to memory loss. A set of diseases are there which affects the performance of human brain like Alzheimer's disease. AD is a life threatening disorder with increasing incidence throughout the world.

Many traditional plants were successfully used for the treatment of memory loss. Though the active principle of various classes of chemical compounds has been isolated from plants, some remain to be identified.

Nowadays, the use of complementary and alternative medicine and especially the consumption of botanicals have been increasing rapidly worldwide, mostly because of the supposedly less frequent side effects when compared to modern western medicine.

Abelmoschus moschatus Medic is a folk medicinal shrub known as Kattukasthuri. In the present study an attempt was made to investigate the Pharmacognostical, Phytochemical screening and Memory Strengthening activity on the Seeds of *Abelmoschus moschatus* Medic.

4. RATIONALE FOR SELECTION OF THE PLANT

Now a day's many plants are being upgraded by validating traditional claims and establishing its medicinal value. Various plants are yet to be scientifically proven for their therapeutic efficacy.

Abelmoschus moschatus Medic. is one such fascinating shrub belonging to the family Malvaceae. This plant is commonly known as musk mallow, Musk recognized by the musk-scented seeds.

The plant has a long history of numerous traditional and ethno botanical applications in diverse cultures. Many tribes considered it has a cure for all ailments. Leaves are used for gonorrhea; roots are used in renal disorders, diuretic, anti spasmodic. In Egypt, seeds are chewed to relieve stomach problems, Infusion, decoction or tincture of seeds used for nervous debility, hysteria and other nervous disorders. Traditionally seeds have been used for treatment of neuro degenerative disorders. [20, 21] The present study was undertaken to investigate the effects of *Abelmoschus moschatus* Medic. on memory strengthening activity.

5. ETHNO BOTANICAL SURVEY

PLANT PROFILE [20]

Plant name : *Abelmoschus moschatus* Medic.

Common name : Musk mallow

Tamil name : Kattukkasturi

Family : Malvaceae

TAXONOMICAL STATUS

Kingdom : Plantae (plants)

Division : Magnoliopsida (flowering agent)

Order : Malvales

Family : Malvaceae

Genus : *Abelmoschus*

Species : *moschatus*

Habitate

Throughout the Philippines and India. In India the plant is grown widely over the Deccan regions, the hilly regions of Karnataka and at the foothills of the Himalayas.

***Abelmoschus moschatus* Medic.**

Figure 5.Whole plant of *Abelmoschus moschatus*

Seeds of *Abelmoschus moschatus* Medic.

Figure 6. Seeds of *Abelmoschus moschatus*

PLANT DISTRIBUTION [21]

Kattukasthuri is an annual, erect and branched herb, about a meter high or less, covered with very long hairs.

DESCRIPTION:

It is annual or biennial, erect herb or under shrub, 0.5-3.5 m tall, hispid, often woody at base with taproot or tuberous root.

Stems : Stem usually solid, sometimes hollow, stem apices and petioles with many obliquely downwardly directed long sharp bristles, rarely sparsely hairy or glabrous.

Leaves : Alternate, extremely variable in shape and size, petiole 6-30 cm long, stipules linear-filiform, simply hairy, lower leaves are orbicular to transversely elliptical in outline, 6-22cm × 8-24cm, base cordate, angular or 3-7 palmately lobed or parted, higher leaves usually narrower and often hastate or sagittate, oblong-lanceolate.

Stipules : Linear to filiform, simply hairy.

Flowers : Bright yellow and large flowers are usually solitary and auxiliary. Epicalyx segments 7-10 rarely more, persistent and linear with simple hairs. Petals obovate, at apex rounded, at base fleshy and ciliate by simple hairs.

Capsule : 5-8cm long, ovoid or globose, occasionally fusiform, somewhat angular, usually hispid by simple, stiff hairs, rarely glabrous, black or dark brown in colour.

Seeds : concentrically ribbed, sub-reniform , mostly glabrous, musky scented, black to brown in colour.

ETHANOBOTANICAL USES [20]

- ✓ Decoction of pounded seed used as a diuretic, tonic and carminatie.
- ✓ Mucilagenous decoction of root and leaves used for gonorrhea.
- ✓ Seeds used as antihysteric.
- ✓ In Malaya, leaves and roots used as poultice.
- ✓ Used for headaches, rheumatism, varicose veins, cystits and fever.

- ✓ In Java, powder or infusion of roots used to stimulate the kidneys and intestines.
- ✓ In the West Indies, seeds are used as antispasmodic.
- ✓ Infusion,decoction or tincture of seeds used for nervous debilty, hysteria and other nervous disorders.
- ✓ In the Carribean, seeds are used for female reproductive problems and for childbirth.
- ✓ In Egypt, seeds are chewed to releive stomach problems, to sooth nerves and "sweeten" the breath,also considered as an aphrodisiac.
- ✓ In Ayurveda, plant considered to pacify aggravated pitta, kapha, bonchitis, asthuma, dyspepsia, coli, calcli, diarrhoea, flatulence, vomiting, nervous sysytem disorders.

6. PLAN OF WORK

I. PHARMACOGNOSTICAL STUDIES

- ✓ Collection of plant material
- ✓ Authentication
- ✓ Macroscopy
- ✓ Microscopy
- ✓ Powder microscopy
- ✓ Physiochemical constants
- ✓ Qualitative analysis of heavy metals and inorganic elements
- ✓ Quantitative estimation of heavy metals and inorganic elements.

II. PHYTOCHEMICAL STUDIES

- ✓ Preparation of extracts
- ✓ Preliminary Phytochemical screening of powder and extracts
- ✓ Fluorescence analysis of powder and extracts
- ✓ Quantitative estimation of phytoconstituents.
- ✓ Thin layer chromatography.
- ✓ High Performance Thin Layer Chromatography.

III. SELECTION OF ACTIVE EXTRACT BASED ON *IN VITRO* ANTI-OXIDANT STUDIES

- ✓ Reducing power Assay
- ✓ Hydrogen peroxide scavenging Assay.

IV. PHARMACOLOGICAL STUDIES

Memory strengthening activity

- ✓ Effect on Transfer Latency
- ✓ Estimation of Brain AchE level.

In vivo Anti oxidant studies

- ✓ Estimation of Brain Malondialdehyde Level.
- ✓ Estimation of Brain Reduced Glutathione Level.

7. METHODOLOGY

7.1 PHARMACOGNOSTICAL STUDIES [35]

Evaluation of a drug means confirmation of its identity, determination of its quality and purity and detection of nature of adulteration. Evaluation of a crude drug can be attempted by different methods which include morphological and microscopical studies of the crude drugs or their physical, chemical and biological behavior. Systematic identification of crude drugs and their quality assurance gives an integral part of drug description.

Pharmacognostical studies basically deals with the identification, authentication and standardization of herbal medicinal plants through organoleptic character, histological character, powder microscopy, quantitative microscopy, histochemical analysis and physio chemical observations as prescribed by an authoritative source such as World Health Organization (WHO).

Plant collection and Authentication

The fresh seeds of the plant *Abelmoschus moschatus* Medic. was collected from Palayamkottai in Tirunelveli Dist, Tamilnadu, India. It was identified and authenticated by the Botanist V.Chelladurai, Research Officer, Botany (Scientist-C), Govt. Council for Research in Ayurveda and Siddha, Govt of India (Retired).

MACROSCOPY

Macroscopical character includes organoleptic characters and morphological features of the plant were studied.

MICROSCOPY [36-43]

Staining method

Fixation of plant organ – The sample or seed was cut fixed in FAA solution (Formalin 5ml + Acetic acid 5ml + 90ml of 70% Ethanol). The specimen was dehydrated after 24 hours of fixing. The seeds were graded with series of tertiary butyl alcohol, as per the standard method.

Infiltration of the specimen - It was carried out by gradual addition of 58 – 60º C of melting pointed paraffin wax until Tertiary butyl alcohol (TBA) solution attained super saturation. The specimens were cast into paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10 - 12µg. Dewaxing of the sections were done by customary procedures. The sections were stained with Toludine blue. Since toludine blue is a polychromatic stain, the sections were stained as per the method published by O'Brein *et al.* [43] The staining results were remarkably good. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to subrein, violet to mucilage and blue to the protein bodies. Whenever necessary sections were also stained with safranin, fast green and iodine for starch.

PHOTO MICROGRAPHS

Microscopic descriptions of tissues were supplemented with photo micrographs whenever necessary. Photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For normal observations bright field was used.

For the study of crystals and lignified cells, polarized light was employed. Since, these structures have birefringent property under polarized light they appear bright against dark background. Descriptive terms of the anatomical features are as given in the standard anatomy books.

POWDER MICROSCOPY

The shade dried, powdered plant material was used for powder microscopic analysis. The organoleptic characters were observed and to identify the different characteristic features various staining reagent were used. Powder was stained with 1% phloroglucinol in 90% ethanol, concentrated hydrochloric acid and observed through microscope. All the lignified cells stained with pink colour. Calcium oxalate crystals were observed under the polarized light microscope.

PHYSIO – CHEMICAL CONSTANTS [44-47]

Shade dried powdered plant material of seeds of ***Abelmoschus moschatus*** **Medic.** was used for the determination of physio chemical constants in accordance with WHO guidelines.

DETERMINATION OF ASH VALUES

Ash values are helpful in determining the quality and purity of a crude drug in the powdered form. The residue remaining after incineration is the ash content of the drug, which simply represents inorganic salts, naturally occurring drug or adhering to it or deliberately added to it, as a form of adulteration.

TOTAL ASH

Total ash is designed to measure the total amount of material remaining after ignition. This includes both physiological ash which is derived from plant tissue itself and non-physiological ash which is the residue of the extraneous matter adhering to the plant surface.

Procedure

Silica crucible was heated to red hot for 30 minutes and it was allowed to cool in desiccators. About 2gm of powdered sample was weighed accurately and evenly distributed in the crucible. Dried at 100 – 105°c for 1 hour and ignited to constant weight in a muffle furnace at 600±25ºC. The crucible was allowed to cool in a desiccator. The percentage of ash with reference to the air dried substance was then calculated.

Water soluble ash

The ash was boiled for 5min with 25ml of water. The insoluble matter was then collected in an ash less filter paper. It was washed with hot water and ignited for 15min at a temperature not exceeding 450°c. The weight of the insoluble matter was subtracted from the weight of the ash and the difference in weight represented the water soluble ash, the percentage of water soluble ash with reference to the air dried substances was calculated.

Acid insoluble ash

Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.

Procedure

To the total ash obtained, 25ml of hydrochloric acid was added, covered with a watch glass and boiled gently for 5min on a burner. The watch glass was rinsed with 5ml of hot water and these washings were added to the crucible. The insoluble matter was collected on an ash less filter paper by filtration and the filter paper was rinsed repeatedly with hot water until the filtrate is neutral and free from acid. Filter paper containing the insoluble matter was transferred to the crucible, dried on a hot plate and ignited to a constant weight in the muffle furnace at 450-500°C. The silica crucible was removed from the muffle furnace and allowed to cool in a desiccator for 30min, and then weighed without delay. The content of acid insoluble ash was calculated.

Sulphated ash

2-3gm of air-dried substance was ignited gently at first in a crucible, until the substance was thoroughly charred. Then the residue was cooled, moistened with 1ml of sulphuric acid, heated gently until the white fumes were no longer evolved and ignited at 800±25°C, until all the black particles were disappeared. The crucible was allowed to cool, a few drops of sulphuric acid was added and heated. Then it was ignited as before, cooled and weighed. The percentage of sulphated ash with reference to the air- dried substance was then calculated.

DETERMINATION OF EXTRACTIVE VALUES

Extractive values are useful for the evaluation of phyto constituents especially when the constituents of a drug cannot be readily estimated by any other means. Further these values indicate the nature of the active constituents present in a crude drug.

Determination of water soluble extractive

5gm of the powder was weighed and macerated with 100ml of chloroform water (95ml distilled water and 5ml chloroform) in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. 2 ml of alcohol was added to the residue and it was dried at 105°C for 1 hour in the hot air oven and cooled in desiccators for 30min and weighed. The process was repeated till a constant weight was obtained; the percentage of water soluble extractive value with reference to the air dried drug was calculated.

Water soluble extractive value = weight of the dried extract / weight of the sample taken × 100

Determination of alcohol soluble extractive

5gm of the powder was weighed and macerated with 100ml 90% ethanol in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. It was dried at 105°C for 1hour in a hot air oven. The dish was cooled in desiccator and weighed. The process was repeated till the constant weight was obtained. The percentage of alcohol soluble extractive value with reference to the air dried drug was calculated.

Alcohol soluble extractive = weight of the dried extract / weight of the sample taken × 100

Determination of non volatile ether soluble extractive (fixed oil content)

A suitably weighed quantity of the drug was transferred to an extraction thimble and extracted with solvent ether or petroleum ether (Boiling Point 40 - 60°C) in a Soxhlet for 6 hours. The extract was filtered into a tarred evaporating dish, evaporated and dried at 105°C to constant weight. The percentage of non volatile ether soluble extractive value with reference to the air dried drug was calculated.

Determination of volatile ether soluble extractive

2gm of powdered drug was accurately weighed and extracted with anhydrous diethyl ether in a continuous extractive apparatus for 20hours. The ether solution was transferred to tarred porcelain dish and evaporated spontaneously. Then it was dried over phosphorous pent oxide for 18hours and the total ether extract was weighed. The extract was heated gradually and dried at 105℃ to constant weight. The loss in weight represents the volatile portion of the extract.

Loss on drying

Specified quantity of the substances was taken in a previously ignited and cooled silica crucible and the substance was evenly distributed by gentle side wise shaking. The crucible with the contents was weighed accurately. The loaded crucible and the lid were placed in the drying chamber (105°C). The substance was heated for a specified period of time to a constant weight. The crucible was covered with the lid and allowed to cool in a desiccator at room temperature before weighing. Finally the crucible was weighed to calculate the loss on drying with reference to the air dried substance.

Determination of foaming index

1gm of the coarsely powdered drug was weighed and transferred to 500ml conical flask containing 100ml boiling water. The flask was maintained at temperature 80-90°C for about 30min. It was then cooled and filtered into a volumetric flask and sufficient water was added through the filtrate to make up the volume to 100ml. The decoction was poured into 10 stopper test tube (height 16cm, diameter 16mm) in successive portions of 1ml, 2ml, 3ml, 4ml up to 10ml and the volume of the liquid in each tube was adjusted with water to 10ml. The tubes were stoppered and shaked in a length wise motion for 15 seconds, two shakes per second. Allowed to stand for 15min and the height of the foam was measured. The results are assessed as follows.

If the height of the foam in every tube is less than 1cm, the foaming index is less than 100. If a height of 1cm is measured in any tube, the volume of the plant material decoction in the tube (a) is used to determine the index. If this tube is the first or second tube in a series, prepare an intermediate dilution in a similar manner to obtain a more precise result.

If the height of the foam is more than 1cm in every tube, the foaming index is over 1000. In this case repeat the determination using a new series of dilution of the decoction in order to obtain a result. Calculate the foaming index using the following formula:

Foaming index = 1000/a

Where, a = the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm is observed.

Determination of swelling index

The swelling index is the volume in ml occupied by the swelling of 1gm of plant material under specified conditions. A specified quantity of the plant material were previously reduced to the required fineness was accurately weighed and transferred into a 25ml glass stoppered measuring cylinder. The internal diameter of the cylinder should be about 16mm, the length of the graduated portion about 125mm, marked in 0.2ml divisions from 0 to 25ml in an upward direction. Unless otherwise indicated in the test procedure, add 25ml of water and shake the mixture thoroughly every 10min for 1hour, allowed to stand for 3 hours at room temperature. The volume in ml occupied by the plant material was measured including any sticky mucilage. Calculate the mean value of the individual determination, related to 1gm of plant material.

QUALITATIVE AND QUANTITATIVE ESTIMATION OF HEAVY METALS AND INORGANIC ELEMENTS [48, 49]

Plant minerals play a vital role in metabolism. Presence of elements vary with the soil, climate conditions etc. There are essential and non essential elements which may be beneficial or harmful to living things. Non essential elements like lead, arsenic, cyanide, chromium, cadmium, aluminium, silver bring about toxic effects resulting in intoxification. Hence, qualitative and quantitative estimation of inorganic elements in the plant *Abelmoschus moschatus* Medic. were carried out.

QUALITATIVE ANALYSIS OF INORGANIC ELEMENTS AND HEAVY METALS [48]

To the ash of the drug material 50%v/v hydrochloric acid was added and kept for 1 hour. It was filtered and the filtrate was used for the following tests.

Aluminium: White gelatinous precipitate of aluminium hydroxide is formed on addition of ammonia solution. It is slightly soluble in excess of the reagent. The precipitate dissolves readily in strong acid and base, but after boiling it becomes insoluble.

Arsenic: Arsenious salts in neutral solution react with solution of copper sulphate to form green precipitate (scheele's green) which on boiling gives a red precipitate of cupric oxide.

Borate: The mixture obtained by the addition of sulpuric acid and alcohol (95%) to a borate when ignited, burns with flame tinged with green.

Calcium: Solution of calcium salts, when treated with ammonium carbonate solution, yield a white precipitate after boiling and cooling the mixture (it is insoluble in ammonium chloride solution).

Carbonate: Carbonate, when treated with dilute acid effervescence, liberating carbon dioxide which is colourless and produces a white precipitate in calcium hydroxide solution.

Chlorides: Chlorides, when treated with silver nitrate solution, yield a white crude precipitate which is insoluble in nitric acid, but soluble after being well washed with water, in diluted ammonia, from which it is re precipitated by the addition of nitric acid.

Copper: An excess of ammonia, added to a solution of a cupric salt, produces first a bluish precipitate and then a deep blue coloured solution.

Iron: Solution of ferric salts, when treated with potassium ferrocyanide solution, yields an intense blue precipitate which is insoluble in dilute Hcl.

Lead: Strong solution of lead salts, when treated with HCL, yield a white precipitate. Which is soluble in boiling water and is re deposited as crystals when the solution is cooled.

Magnesium: Solution of magnesium salts, when treated with ammonium carbonate solution and boiled, yield a white precipitate, but yield no precipitate in the presence of ammonium chloride solution.

Mercury: Solution of mercury salts, when treated with sodium hydroxide solution, yields a yellow precipitate.

Nitrate: With solution of ferrous sulphate no brown colour was observed but if sulphuric acid is added (slow from the side of the test tube), a brown colour is produced at the junction of two liquids, indicating the presence of nitrates.

Phosphate: Solution of phosphate when treated with silver nitrate with dilute ammonia solution and in dilute nitric acid yield yellow precipitate of normal silver ortho phosphate (distinction from meta and pyrophosphate) solution.

Potassium: Moderately strong potassium salts, which have been previously ignited to remove ammonium salts, when treated with perchloric acid (60%) yield a white crystalline precipitate.

Silver: Solution of silver salts, when treated with potassium iodide solution yield a cream coloured precipitate which is insoluble in dilute ammonia solution and in nitric acid.

Sulphates: Solution of sulphates, when treated with lead acetate solution yields a white precipitate which is insoluble in ammonium acetate solution and in sodium hydroxide.

QUANTITATIVE ESTIMATION OF INORGANIC ELEMENTS: [50]

Inductive coupled plasma-Optical emission spectroscopy (ICP-OES)

It is an excellent multi-element technique with relatively good sensitivity and selectivity when configured correctly. This technique utilizes the plasma as an ion source or light emission source are capable of producing values.

QUANTITATIVE ANALYSIS OF HEAVY METALS

Instrumentation parameters:

Instrument name: Inductive coupled plasma-Optical emission spectroscopy

Instrument Model: PE Optima 5300DV ICP-OES; Optical system Dual view-axial or radial

Detector system: Charge coupled detector, (UV-Visible detector which is maintaining at -40º C) to detect the intensity of the emission line.

Light source (Torch): Positioned horizontally in the sample compartment along the central axis of the spectrometer optics. Changing from axial to radial viewing is a simple software command and is accomplished by computer control of a mirror located in the optical path. The torch assembly of this system comprises of two concentric quartz tubes.

Standard alumina injector: 2.0mm inner diameter.

Spray chamber: Scott type

Nebulizer: Cross flow gem tip.

Preparation of sample by acid digestion method:

50mg of powder was treated with acid mixture of sulphuric acid: water in the ratio of 4:1in the Kjeldahl flask and heated continuously till the solution is colourless. The sample mixture was then transferred in a 25ml volumetric flask and made up to the volume with distilled water. Blank solution was prepared as above without sample.

The standards of Arsenic, Lead, Mercury and Cadmium were prepared as per the protocol and the calibration curve was developed for each of them.

Detection:

Samples were analyzed for the detection and quantification of the calcium, sulphate, borate, silver, aluminium, copper, potassium, chloride by Inductively Coupled Plasma Emission Spectrometry.

7.2 PHYTOCHEMICAL STUDIES

Phytochemical evaluation is used to determine the nature of phyto constituents present in the plant by using suitable chemical tests. It is essential to study the pharmacological activities of the plant. It can be done by confirmation with different chromatographic techniques like TLC and HPTLC. Therefore a complete investigation is required to characterize the Phyto constituents qualitatively and quantitatively.

Preparation of Extracts [51]

Extraction is the preliminary step involved in the phytochemical studies. It brings out the metabolites into the extracting solvent depends upon its polarity.

Extraction

The first step was the preparation of successive solvent extracts. The dried coarsely powdered sample of *Abelmoschus moschatus* seed (500gm) was first extracted with Hexane (60-80°C) in Soxhlet apparatus and then with solvents of increasing polarity like ethyl acetate and ethanol at 60 - 70ºC. Each extract was concentrated using rotary vacuum evaporator. The percentage yield, colour and consistency of these extracts were recorded and preceded for further detailed phyto chemical and pharmacological screening.

PRELIMINARY PHYTOCHEMICAL SCREENING [52-54]

The chemical tests for various Phyto constituents in the dried powder and extracts of seeds of *Abelmoschus moschatus* were carried out as described below and the results were recorded.

1. **Detection of Alkaloids**

- Dragendorff's reagent :

  The substance was dissolved in 5ml of distilled water, to this 5ml of 2M HCL was added until an acid reactions occurs, then 1ml of Dragendorff's reagent was added and examined for an immediate formation of an orange red precipitate.

- Mayer's reagent:

  The substance was mixed with little amount of dilute hydrochloric acid and Mayer's reagent and examined for the formation of white precipitate.

- Wagner's reagent :

  The test solution was mixed with Wagner's reagent and examined for the formation of reddish brown precipitate.

2. **Detection of Glycosides**

- Borntrager's test :

  The powdered material was boiled with 1ml of sulphuric acid in a test tube for five minutes. Filtered while hot, cooled and shaken with equal volume of chloroform. The lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammonical layer.

- Modified Borntrager's test :

  The test material was boiled with 2ml of the dilute sulphuric acid. This was treated with 2ml of 5% aqueous ferric chloride solution (freshly prepared) for 5 minutes, and shaken with equal volume of chloroform. The lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammonical layer.

3. Detection of Steroids and Triterpenoids

- Libermann Burchards Test:

  The powdered drug was treated with few drops of acetic anhydride, boiled and cooled. Conc.sulphuric acid was added from the sides of the test tube, brown ring is formed at the junction of two layers and upper layer turns green which shows presence of steroids and formation of deep red color indicates presence of tri terpenoids.

- Salkowski Test :

  The extract was treated with few drops of concentrated sulphuric acid, red color at lower layer indicates presence of steroids and formation of yellow colored lower layer indicates presence of tri terpenoids.

4. **Detection of Flavonoids**

- Shinoda test :

  To the solution of extract, few piece of magnesium turnings and concentrated Hcl was added drop wise, pink to crimson red, occasionally green to blue color appears after few minutes indicates the presence of flavonoids.

- Alkaline reagent test :

  To the test solution few drops of sodium hydroxide solution was added, intense yellow color is formed which turns to colorless on addition of few drops of dilute acid indicate presence of flavonoids.

5. **Detection of Carbohydrates**

- Molisch's test :

  To the test solution few drops of alcoholic alpha napthol and few drops of conc. sulphuric acid were added through the sides of test tube, purple to violet color ring appears at junction.

- Fehling's test :

  The test solution was mixed with Fehling's I and II and heated and examined for the appearance of red coloration for the presence of sugar.

6. **Detection of Phenols**

- Ferric chloride test :

  A small quantity of substance were dissolved with 2ml distilled water and a few drops of 10% aqueous ferric chloride solution was added and observed for appearance of blue or green color.

7**. Detection of Proteins**

- Biuret test :

  The sample was treated with 5-8 drops of 10% w/w copper sulphate solution, violet color is formed.

8. **Detection of Tannins**

- Lead acetate test :

  The test solution was mixed with basic lead acetate solution and examined for formation of a white precipitate.

- Ferric chloride test :

  A few drops of 5% aqueous ferric chloride solution was added to 2ml of an aqueous extract of the drug and examined for the appearance of bluish black color.

9. **Detection of Saponins**

- A drop of sodium bicarbonate solution was added to the sample and the mixture was shaken vigorously and left for 3 minutes. Development of any honey comb like froth was examined.

10. **Detection of Gum and Mucilage**

- Small quantities of test substances was dissolved in 5 to 10ml of acetic anhydride by means of heat, cooled and add 0.05ml of concentrated sulphuric acid; it is examined for the formation of bright purplish red color.

11. Detection of fixed oils and fats:

- Small quantities of extracts were pressed between two filter papers. An oily stain on filter paper indicates the presence of fixed oils and fats.

FLUORESCENT ANALYSIS [55, 56]

Fluorescence analysis was carried out in day light and in UV light. The leaf powder and extracts were treated with different solvents and the fluorescence was observed in day light and in near and far UV light.

QUANTITATIVE ESTIMATION OF TOTAL PHENOLIC AND TOTAL FLAVONOID CONTENT [57, 58, 59]

Total phenolic content (Folin – Ciocalteu's assay)

Total phenolic content of the extracts were determined using Folin –Ciocalteu's assay. 0.5ml extract solutions were mixed with 2.5ml of 10 fold diluted Folin Ciocalteu's reagent and 2.5ml of 7.5% sodium carbonate. After incubation at 40°c for 30 minutes, the absorbance of the reaction mixtures was measured at 765nm in a spectrophotometer. Three replicates were made for each test sample. Gallic acid was used as a standard and total phenolic content of the extract was expressed in mg of Gallic acid equivalents (mg GAE/g extract).

Total Flavanoid content

Total flavanoid content was determined by calorimetric method, using quercetin as a standard. The test samples were individually dissolved in DMSO. Then the sample solution (150 µl) was mixed with 150 µl of 2% aluminium chloride. After 10min of incubation at ambient temperature, the absorbance of the supernatant was measured at 435nm using spectrophotometer. Three replicates were made for each test sample. The total flavanoid content was expressed as quercetin equivalent in mg/gm extract (mg QRT/gm extract).

CHROMATOGRAPHY [60-63]

Chromatography methods are important analytical tool in the separation, identification and estimation of components present in the plant.

THIN LAYER CHROMATOGRAPHY

Principle

Thin layer chromatography is a technique used for the separation, identification and estimation of single or mixture of components present in the various extracts. It is reliable technique in which solute undergoes distribution between two phases, stationary and mobile phase. The separation is mainly based on the differential migration that occurs when a solvent flows along the thin layer of stationary phase. This may be achieved by partition and adsorption depending on stationary phase used.

TLC Plate Preparation

The plates were prepared using Stahl TLC spreader. 40gm of silica gel G was mixed with 85ml of water to prepare homogenous suspension and poured in the spreader. 0.25mm thickness of plates was prepared, air dried until the transparency of the layer disappeared, then dried at 110°C for 30 minutes and kept in desiccators.

Selection of mobile phase

Solvent mixture was selected on the basis of the phyto constituents present in each extract. Factors such as nature of components, stationary phase, mobile phase, polarity, influence the rate of separation of constituents. From the vast analysis, best solvent was selected which showed good separation with maximum number of components.

HPTLC- FINGERPRINT PROFILE

HPTLC is one of the versatile chromatographic method which helps in the identification of compounds and thereby authentication of purity of herbal drugs. The time required in this method for the demonstration of most of the characteristic constituents of a drug is very quick and short. In addition to qualitative detection, HPTLC also provides semi-quantitative information on major active constituents of a drug, thus enabling an assessment of drug quality.

HPTLC serves as a convenient tool for finding the distribution pattern of phyto constituents which is unique to each plant. The fingerprint obtained is suitable for monitoring the identity and purity of drugs and for detecting adulteration and substitution. HPTLC technique is helpful in order to check the identity, purity and standardize the quantity of active principles present in the herbal extract.

Instrument Conditions:

Sample used **:** Ethanol Extract

Instrument : CAMAG HPTLC

HPTLC Applicator : CAMAG LINOMAT IV

HPTLC Scanner : CAMAG TLC SCANNER II

Sample dilution : 100mg of sample extracted with 1ml of Ethyl acetate

Volume of injection : 20µl

Mobile phase : Ethyl acetate : Hexane (6:4)

Lambda max : 254nm

Lamp : Deuterium

Stationary phase : TLC silica gel 60 F_{254} (Merck)

Equipment:

A Camag HPTLC system equipped with a sample applicator Linomat IV, Twin trough plate development chamber, TLC Scanner II.

Chromatographic conditions:

The estimation has been done using the following chromatographic conditions. Chromatography was performed on a 10 × 10cm pre-activated HPTLC silicagel 60 F_{254} plate. Samples were applied to the plate as 6mm wide band with an automatic TLC applicator Linomat IV with nitrogen flow (CAMAG, Switzerland), 8mm from the bottom. Densitometric scanning was performed on CAMAG scanner II. The plates were pre-washed with solvent ethyl acetate.

SELECTION OF ACTIVE EXTRACT

IN VITRO ANTI OXIDANT ACTIVITY [64, 65]

There has been a lot of interest in the development of alternative medicine for memory impairment. Oxidative stress plays a major role in the progression of memory impairment. It occurs when the balances between free radicals which attack nerve cells and anti oxidants which protect them are lost.

Anti oxidants like poly phenols and flavanoids are therefore very helpful in maintaining the brain function and improving memory. It has become evident that flavanoids are able to exert neuro protective actions even at low concentration via their interactions with critical neuronal intracellular signaling pathways pivotal in controlling neuronal survival and differentiation, long term potentiation and memory.

Therefore it can be concluded from various studies that anti oxidants alone can also be helpful in enhancement of memory.

In vitro antioxidant activities for the extracts were carried out using the following methods.

1. Reducing power assay
2. Hydrogen peroxide scavenging assay.

REDUCING POWER ASSAY

The reducing power assay was determined according to the method of Oyaizu. Various concentrations of plant extracts (1ml) were mixed with 1ml of 200 mmol/lt of sodium phosphate buffer (pH 6.6) and 1ml of 1% potassium ferricyanide. The mixture was incubated at 50ºc for 20mins. To this 1ml of 10% tri chloro acetic acid (w/v) was added and the mixture was centrifuged at 2000rpm for 10mins. The upper layer (2.5ml) was mixed with 2.5ml of deionised water and 0.5ml of fresh ferric chloride (0.1%). The absorbance was measured at 700nm. A higher absorbance value indicates a higher reducing power.

HYDROGEN PEROXIDE SCAVENGING ASSAY

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Ruch. A solution of Hydrogen peroxide (2mmol/l\lt) was prepared in phosphate buffer (PH 7.4). Various concentrations of extracts (10-100µg/ml) were added to hydrogen peroxide solution (0.6ml). Absorbance at 230nm was determined after 10min against a blank solution containing phosphate buffer without hydrogen peroxide.

% scavenging activity = Abs (control) - Abs (standard) / Abs (control) × 100

7.4 PHARMACOLOGICAL STUDIES

IN-VIVO MEMORY STRENGTHENING ACTIVITY

In *in-vitro* method, the ethanolic extract showed potent anti oxidant activity which was comparable with the standard than ethyl acetate extract. Hence Ethanolic extract was selected for *in-vivo* study.

Materials and Methods

Plant extract

Ethanolic extract of seeds of *Abelmoschus moschatus* Medic.

Drugs and Chemicals

- ✓ 0.1M Phosphate buffer
- ✓ Sodium Hydroxide
- ✓ 5,5'-dithiobis nitro benzoic acid (DTNB)
- ✓ Acetyl thio choline iodide

Preparation of drug solutions

DTNB was prepared using 0.1M phosphate buffer. Acetyl thio choline iodide was dissolved in 1 ml of distilled water.

Animal selection and procurement

Healthy young Swiss-Albino mice (weighing about 20-30gm) were procured from the Madras Medical College animal house. The animals used for the entire study was approved by the Institutional Animal Ethical Committee which is certified by the Committee for the purpose of control and supervision of experiments on animals, India.

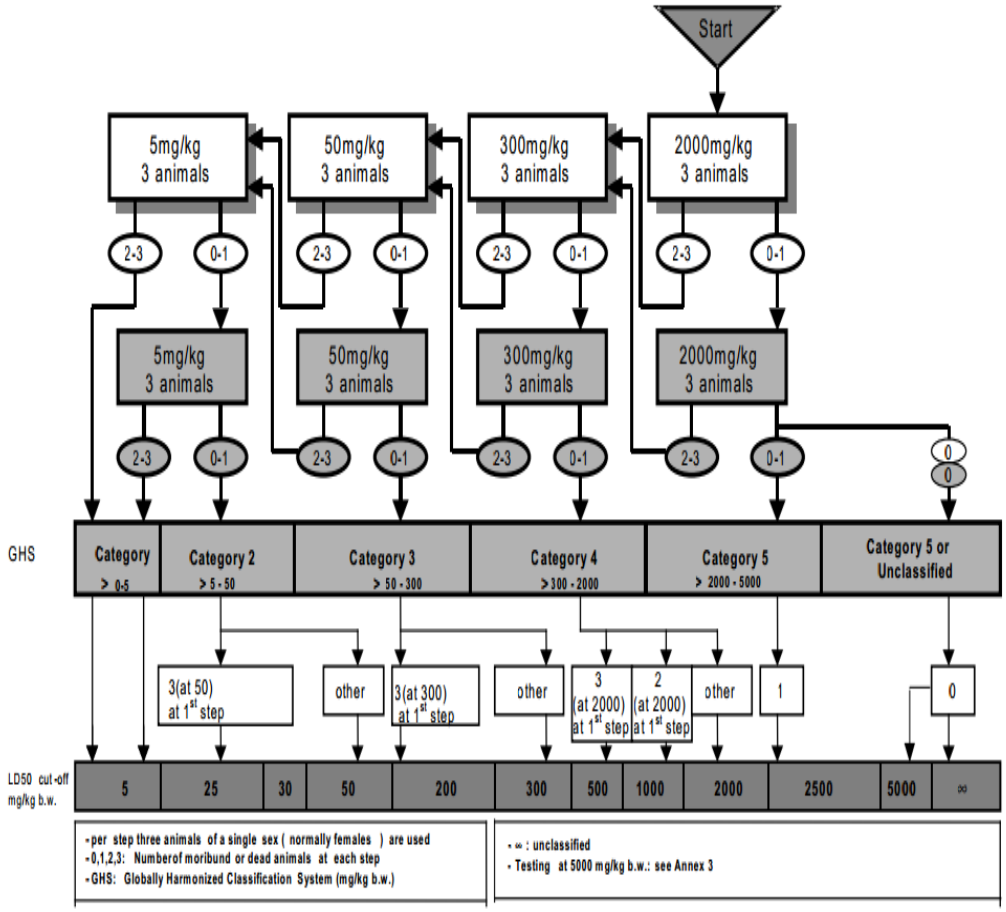
Approved CPCSEA Registration No: 10/243/CPCSEA Dated: 22/11/13.

The procured animals were kept in a clean, dry polycarbonate cages and maintained in a well-ventilated animal house. The temperature of experimental animal room was maintained at 22ºC (± 3ºC) and the relative humidity was maintained from 50-60%. Lighting was artificially maintained for 12hrs dark and 12hrs light. All the animals were kept in the cages for at least 5days prior to dosing for acclimatization to the laboratory conditions. The animals were fed with standard pellet diet and water was given ad libitum. Before starting the dose, the animals were fasted overnight but allowed to access water.

Acute oral toxicity studies [(66)]

The acute oral toxicity study was carried out for ethanolic extract using OECD guidelines 423 (Organization of Economic Co-operation and Development). A single dose of 2000mg/kg of the extract p.o was given and this was used as a starting dose. After oral administration, the animals were observed every 1 hour for 24 hrs to assess the general behavioral changes and mortality. They were further observed for 72 hrs for toxic symptoms and mortality. After 72hrs, no toxic signs were observed. So, $1/10^{th}$ and $1/5^{th}$ of the dose (200 and 400mg/kg) were selected for this study.

ANNEX 2d: TEST PROCEDURE WITH A STARTING DOSE OF 2000 MG/KG BODY WEIGHT



13/14

Figure 7.Acute oral toxicity studies

EXPERIMENTAL DESIGN

MEMORY STRENGTHENING ACTIVITY

- ✓ Effect on Transfer Latency
- ✓ Estimation of brain Acetyl cholinesterase level.

Method: Elevated plus maze model

Animals: Swiss albino mice

Grouping of animals

Animals were divided into 5 groups of six animals each.

Group I: Vehicle control- Distilled water administered orally for 7days, after 90min of administration, Transfer latency (TL) was recorded. Retention of learned task was examined after 24hrs.

Group II: Diazepam (1mg/kg) was injected before training. TL was recorded after 45min of injection. Retention was examined after 24hrs.

Group III: Piracetam (200mg/kg) was injected for 7days and on the 7^{th} day after 90min of drug administration, diazepam (1mg/kg) was given i.p. TL was recorded after 45min of injection and after 24hrs.

Group IV: Test drug I (200mg/kg) was given orally for 7 days and on 7^{th} day after 90min of drug administration, diazepam (1mg/kg) was given i.p. TL was recorded after 45min of injection and after 24hrs.

Group V: Test drug II (400mg/kg) was given orally for 7 days and on 7^{th} day after 90min of drug administration, diazepam (1mg/kg) was given i.p. TL was recorded after 45min of injection and after 24hrs.

Elevated plus maze model [67, 68]

Figure 8.Elevated plus maze model

The elevated plus-maze consisting of two open arms (16×5cm) and two enclosed arms (16×5×12 cm) was used. The maze was elevated to height of 25cm. Mice were placed individually at the end of an open arm facing away from central platform and the time taken to move from the end of the open arm to either of closed arm was recorded(Transfer Latency, TL). If the animal did not enter into one of the enclosed arms within 90sec, it was gently pushed into one of the two enclosed arms the TL was assigned as 90sec. The mice was allowed to explore the maze for another 10sec and then returned to its home cage. Retention of this learned task was examined after 24hrs, after the first day trial (i.e. on 8th day)

On the 9th day animals in all the groups were sacrificed by cervical dislocation and the brains were removed and used for the estimation of Acetyl cholinesterase, Malondialdehyde and Reduced Glutathione.

PREPARATION OF BRAIN HOMOGENATE

The brains of the animals were removed quickly and placed in ice cold saline. The tissues were weighed and homogenized in 0.1M phosphate buffer (pH-8) and the brain homogenate was used for the estimation of Brain AchE level.

Neurotransmitter plays a main role in maintaining normal function and behaviour of brain. Acetylcholine is one of the most important neurotransmitter in the brain. It plays a large role in learning and memory. An imbalance in acetylcholine can lead to learning and memory problems.

Estimation of Brain Acetyl cholinesterase level [69, 70]

Estimation of brain AchE level provides a relatively easy and valuable assessment of cholinergic function. The method of AchE activity estimation is popularly known as Elman's method named after George Ellman who developed this method in 1961(Ellman et al., 1961).

The esterase activity was measured by providing an artificial substrate, acetylthiocholine (ATC). Thiocholine released because of the cleavage of ATC by AchE was allowed to react with the –SH reagent 5, 5'-dithiobis nitro benzoic acid, which is reduced to thio nitro benzoic acid, a yellow coloured anion with an absorption maxima at 412nm.

ASSAY PROCEDURE [71]

- ✓ 0.4ml of aliquot of brain homogenate was added to a cuvette containing 2.6ml of phosphate buffer (0.1M) and to this 100µl of DTNB was added.
- ✓ The contents of the cuvette were mixed thoroughly by bubbling air and absorbance was measured at 412nm in spectrophotometer, when absorbance reaches a stable value, it was recorded as the basal reading.
- ✓ 100µl of substrate (ATC) was added and change in absorbance was recorded for a period of 10mins at intervals of 2mins. Change in the absorbance per minute was determined.

The enzyme activity was calculated using the following formula

$R = 5.74 (10^{-4}) \Delta A/Co,$

R = Rate in moles substrate hydrolyzed per min per gm of tissue,

ΔA = Change in absorbance per min,

Co = Original concentration of tissue (mg/ml).

IN VIVO ANTIOIXDANT ACTIVITY

The brain homogenate was used for the estimation of

1. Brain Malondialdehyde.
2. Brain Reduced Glutathione

ESTIMATION OF BRAIN MALONDIALDEHYDE [72, 73]

Malondialdehyde, indicator of lipid per oxidation was determined as described by Ohkawa et al. The reaction mixture consist of 0.2ml of 8.1% sodium lauryl sulphate, 1.5ml of 20% acetic acid (pH 3.5) and 1.5ml of 0.8% aqueous solution of thio barbituric acid was added to the 0.2ml of processed brain homogenate. The mixture was made up to 4ml with distilled water and heated at 95ºC for 60 minutes. After cooling with tap water, 5ml of n-butanol and pyridine (15:1v/v) and 1ml of distilled water was added and centrifuged. The organic layer was separated out and the absorbance was measured at 532nm using UV-Visible spectrophotometer.

ESTIMATION OF BRAIN REDUCED GLUTATHIONE (GSH) [72]

GSH estimation in brain homogenate was measured according to the Ellman method. This method is based on the development of a yellow colour when 5, 5’ di thio bis nitro benzoic acid (DTNB) is added to the compound containing the sulfydryl groups. To 0.5ml of brain homogenate, 1.5ml of 0.2ml of Tris buffer (PH-8.2) and 0.1ml of 0.01M DTNB were added and this mixture was brought to 10ml with 7.9ml of methanol. The above reaction mixture was centrifuged at 1000 rpm for 15minutes, collect the supernatant liquid. The absorbance of supernatant was read in a spectrophotometer against reagent blank (without sample) at 412nm.

8. RESULTS AND DISCUSSION

PHARMACOGNOSTICAL STUDIES:

The results of the pharmacognostical studies are as follows.

Organoleptic characters

Nature - Brownish black coarse powder

Colour - Brown

Odour - Musk like odour

Taste - Characteristic

Morphological Features:

Colour - Grayish Brown and Blackish in colour.

Odour - Musk like odour on crushing the seeds.

Appearance - Hard seed coat.

Shape - Reni form

Seed length - 0.4 -0.5cm

Seed width -0.5 - 0.7cm

Figure 9.Macroscopy of seeds

Microscopic features

Transverse section of seed

- ✓ A typical Malvaceous seed which is dicot and bitegmic (Angiosperms with two integuments)
- ✓ The seed composed of epidermis, endosperm and embryo.
- ✓ Testa is composed of 8 – 10 layers of cells in three different zones.
- ✓ Outer zone consist of epidermal cells which are polygonal with thickened walls.
- ✓ Middle zone consist of tangentially elongated compact cells with brown colour pigments nearly 3-4 layers.
- ✓ Inner zone is composed of colourless parenchyma cells in 2-3 cell layers. Inner zone is followed by Palisade like scelerides layer comprises of radially elongated cells.
- ✓ Endosperm is single layered contain bands of pigmented cells intermitted with pockets of mucilaginous cells.
- ✓ Endosperm is followed by gland dotted embryo with folded cotyledons.

MICROSCOPIC FEATURES OF THE SEEDS

TRANSVERSE SECTION OF THE SEED

Testa shows three different zones (Outer zone, Middle zone, Inner zone)

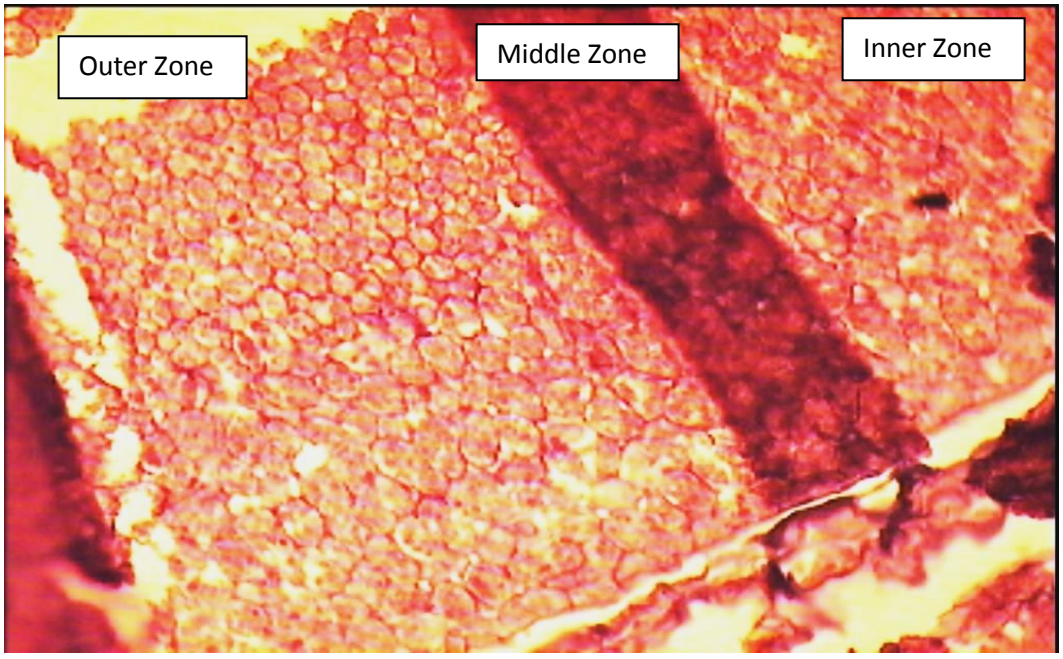


Figure 10. T.S of seed

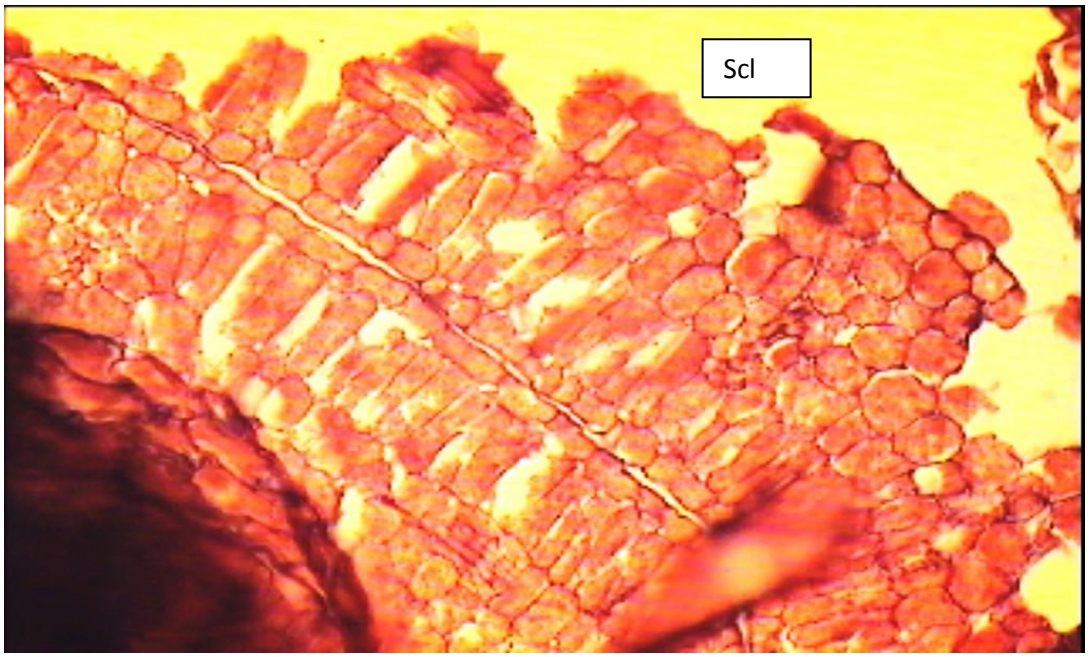


Figure 11.Palisade like Sclereid layer (scl)

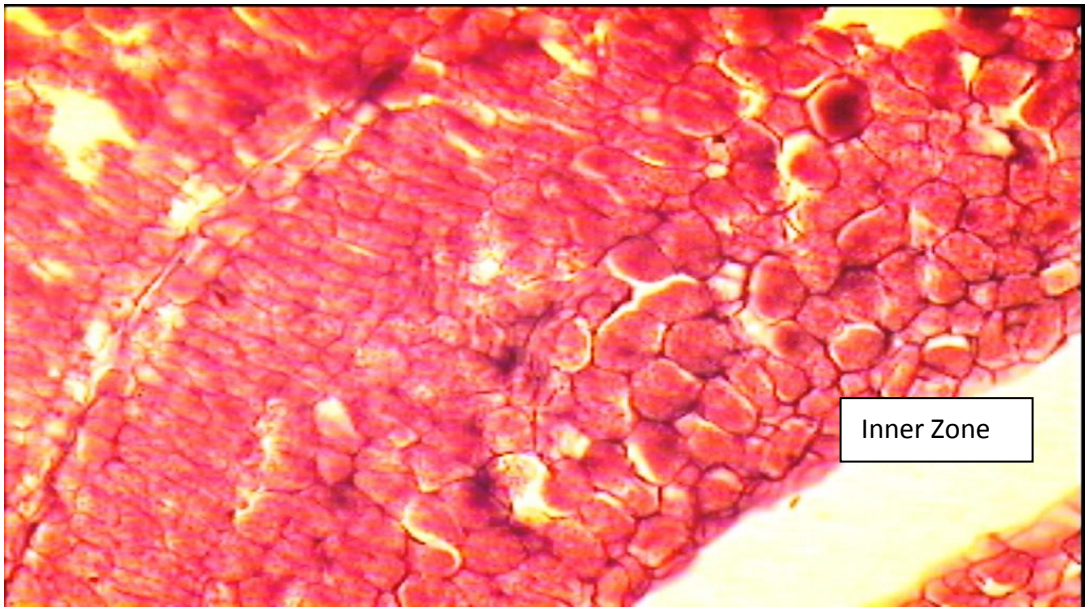


Figure 12.Inner zone of the seed

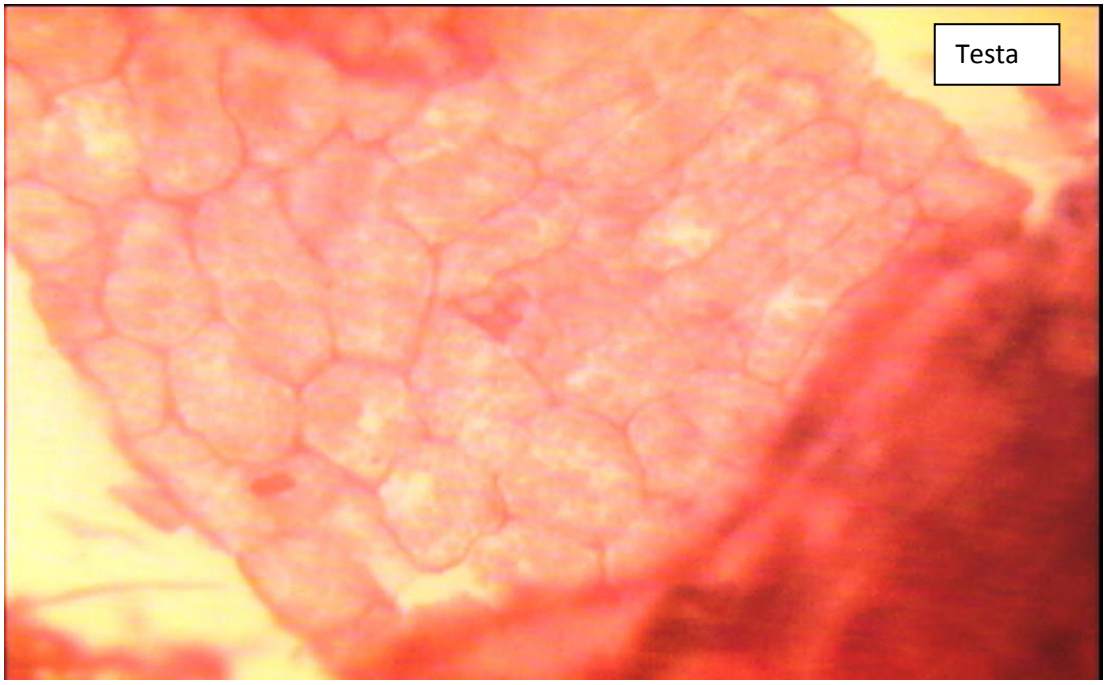


Figure 13.Inner zone of testa

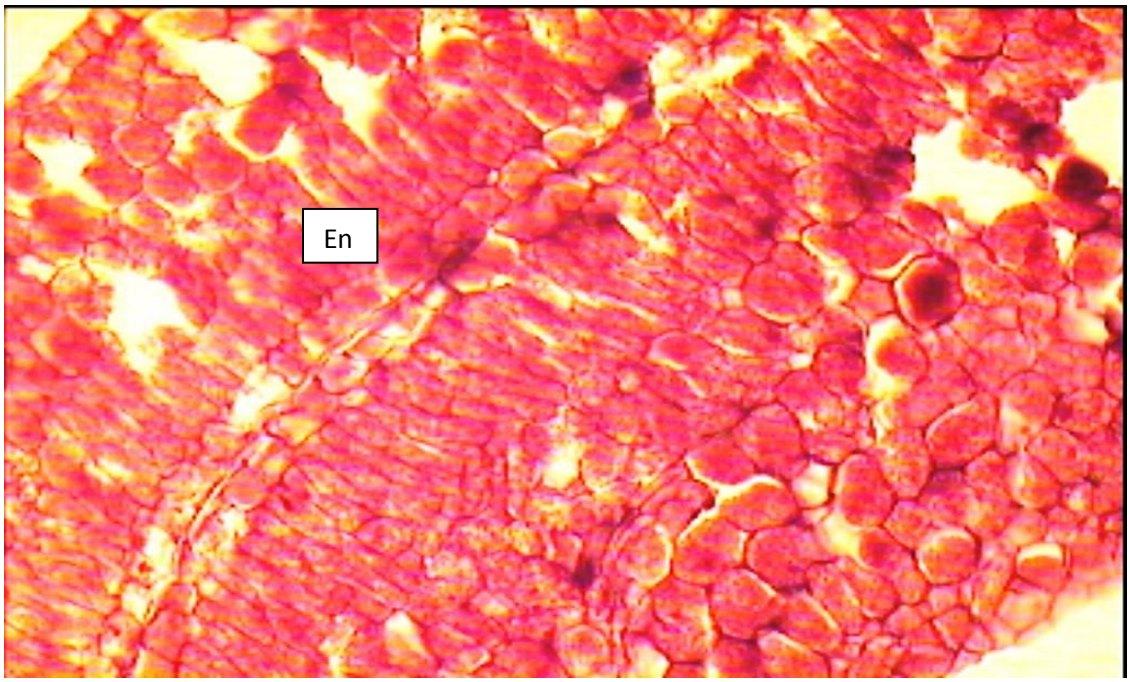


Figure 14. Endosperm layer contains pigmented cells (En)

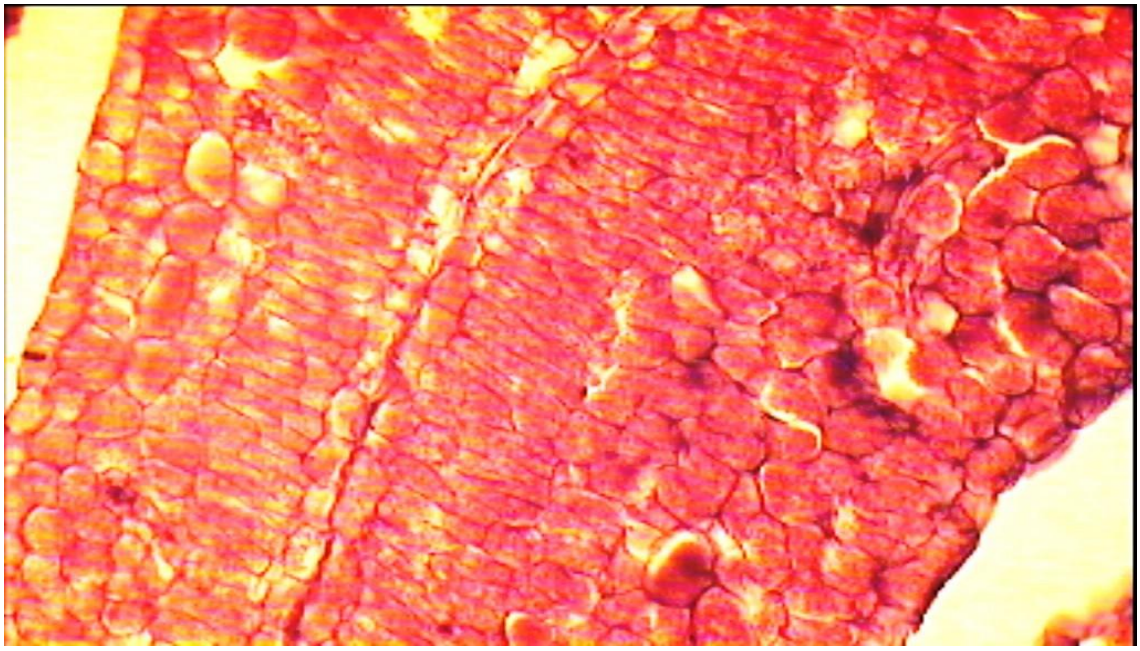


Figure 15. Middle zone consist of cells with brown colour pigment

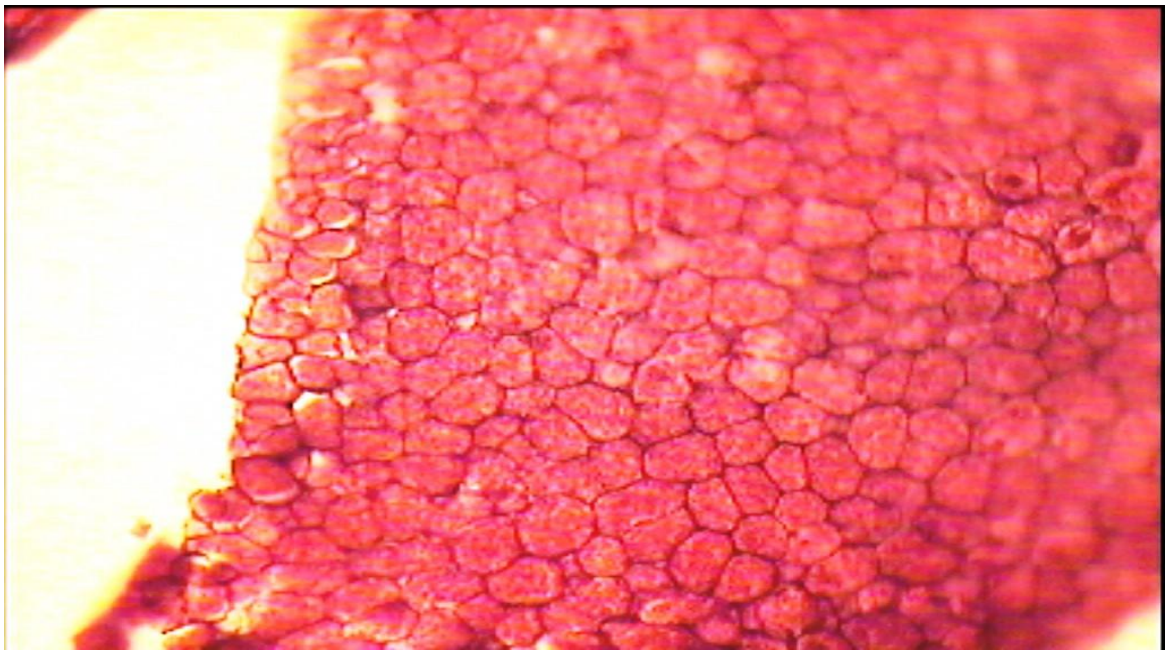


Figure 16.Embryo with folded cotyledons

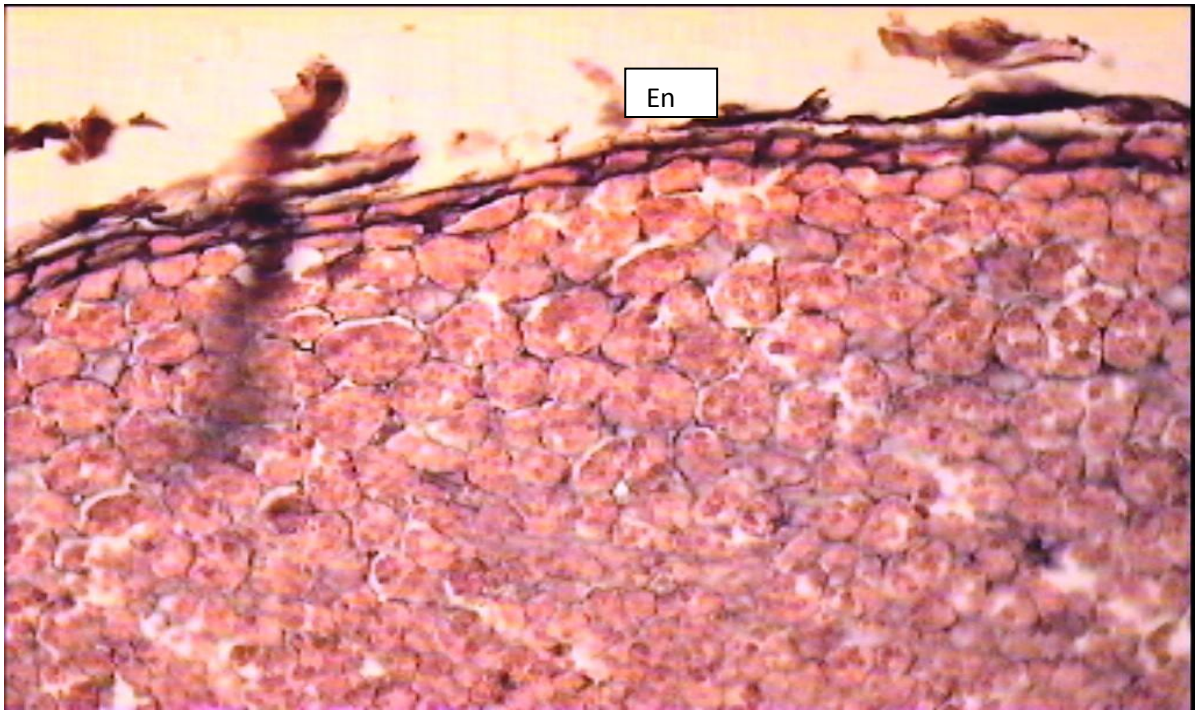


Figure 17.Single layered Endosperm cells (En)

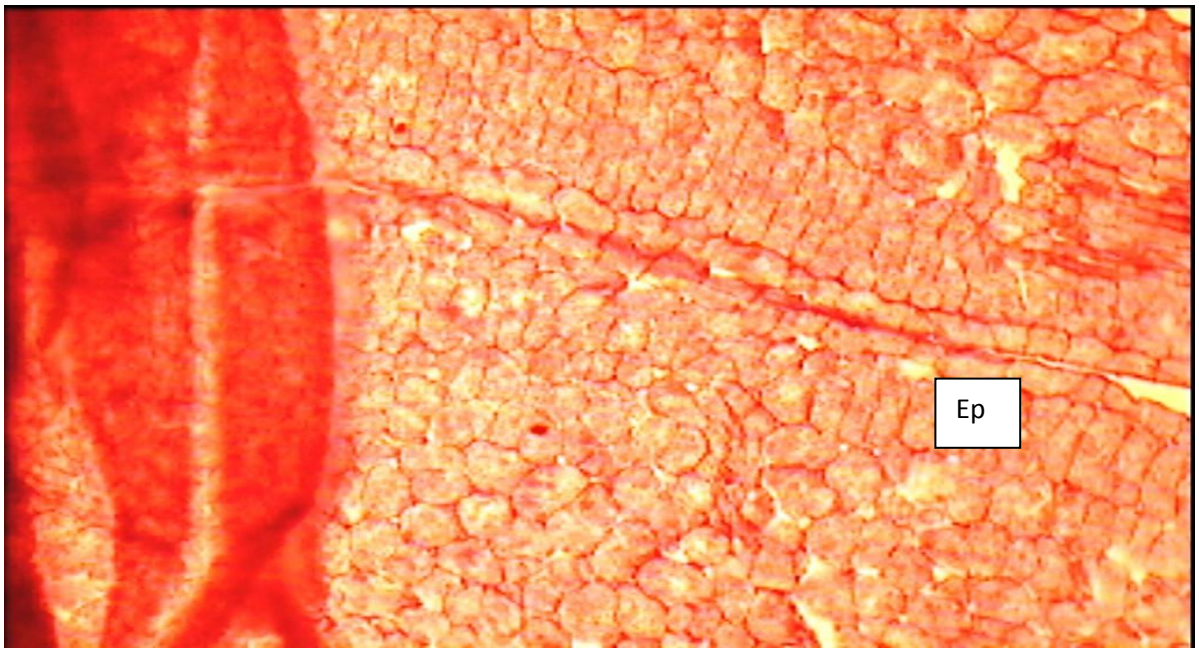


Figure 18.Outer zone with Polygonal epidermal cells (Ep)

POWDER MICROSCOPY

The powder microscopy of seeds of *Abelmoschus moschatus* Medic. showed the presence of sclerenchyma, mucilage cells, parenchyma and oil globules.

Figure 19.Scelrenchyma cells

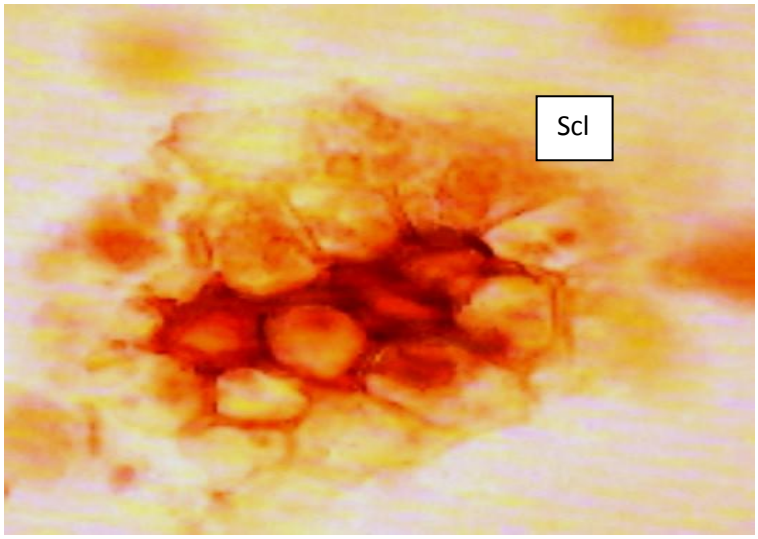


Figure 20.Mucilage cells

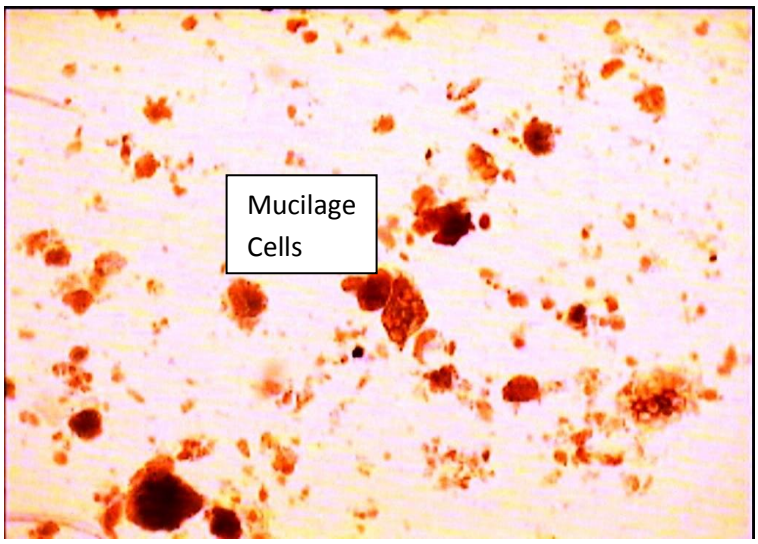


Figure 21.Testa

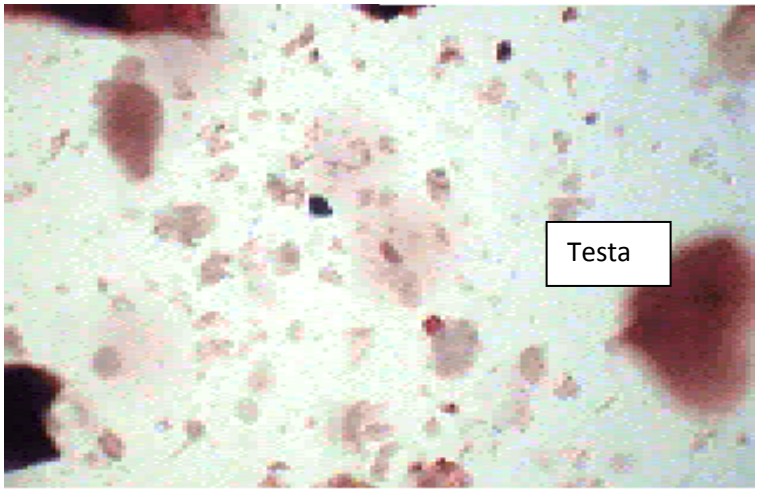


Figure 22.Parenchyma and sclerenchyma cells

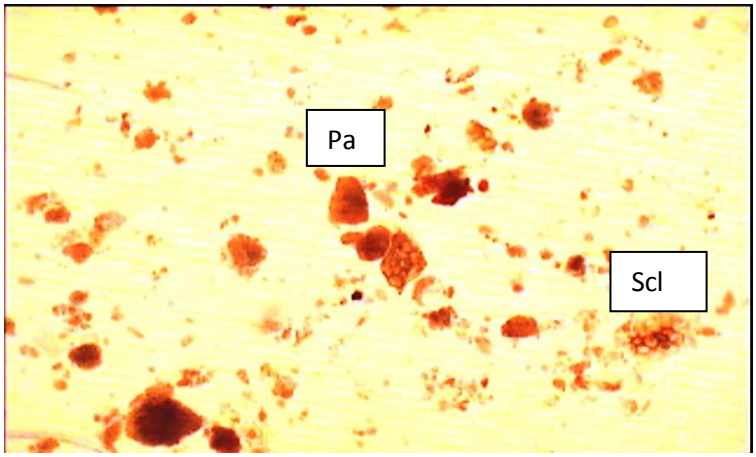


Table 2.The Physicochemical analysis of the seeds of *Abelmoschus moschatus* Medic.

S.NO	PHYSIO-CHEMICAL CONSTANTS	RESULTS (%w/w)
I	**ASH VALUE**	
1.	Total ash	9.66±1.53
2.	Water soluble ash	6.83±0.36
3.	Acid insoluble ash	7.56±0.75
4.	Sulphated ash	10.95± 0.41
II	**EXTRACTIVE VALUE**	
1.	Water soluble extractive	8.35±0.41
2.	Alcohol soluble extractive	7.95±0.56
3.	Ether soluble extractive	4.61±0.61
4.	Non volatile ether soluble extractive	4.31±0.59
III	**Loss on drying**	2.50±0.78
IV	**Crude fibre content**	11.5±0.89
V	**Foaming index**	<100
VI	**Swelling index**	NIL

Values are expressed as Mean ± SD, n=3

INORGANIC ELEMENTS AND HEAVY METAL ANALYSIS

Qualitative and quantitative estimation of inorganic metals were analyzed using Inductive coupled plasma analysis method and the results were tabulated as follows.

Table 3.Qualitative estimation of inorganic elements of *Abelmoschus moschatus* Medic.

S.No	INORGANIC ELEMENTS	OBSERVATIONS
1.	Aluminium	+
2.	Chloride	+
3.	Copper	+
4.	Calcium	+
5.	Iron	-
6.	Borate	+
7.	Potassium	+
8.	Carbonate	-
9.	Sulphates	+
10	Silver	+

Table 4.Quantitative estimation of inorganic elements of *Abelmoschus moschatus*.

S.NO	INORGANIC ELEMENTS	TOTAL AMOUNT (%W/W)
1	Calcium	0.57
2	Sulphate	1.48
3	Borate	0.96
4	Silver	0.26
5	Aluminium	1.68
6	Copper	1.28
7	Potassium	2.20
8	Chloride	1.46

Quantitative estimation of Heavy metals by ICP OES method

The quantification of the individual heavy metals was analyzed for the powdered mixture of *Abelmoschus moschatus* Medic. by ICP-OES technique the following metals like arsenic, lead, cadmium were detected and quantified, results are given in the following table

Table 5.Quantitative estimation of Heavy metals

S.No	Element	Results (ppm)	Specification
1.	Mercury	Not detected	Not more than 0.5ppm
2.	Arsenic	0.084	Not more than 5.0ppm
3.	Lead	0.045	Not more than 10ppm
4.	Cadmium	0.041	Not more than 0.3ppm

The above observation showed that the material is within the limits as per WHO standard and it is safe to consume internally.

DISCUSSION

Pharmacognostical studies play a key factor in establishing the authenticity of the plant material. The botanical identity of the seed was established by examining its anatomical features.

Abelmoschus moschatus Medic is the sole number of its genus and is closely related to temperate family Malvaceae. The anatomical examination of *Abelmochus moschatus* seeds exhibited important microscopical features like endosperm, embryo and parenchymatous cells.

Testa showed three zones like outer zone, middle zone and inner zone. The outer zone consists of epidermal cells which are polygonal with thickened walls. Middle zone consist of tangentially elongated compact cells with brown colour pigments, Inner zone consist of colourless parenchyma.

Powder microscopy showed the presence of parenchyma, sclerenchyma and mucilage cells, indeed these features can be employed for inter specific identity of drugs.

Physiochemical parameters are mainly used in judging the purity and quality of the powdered drug. Ash values of a drug give an idea of the earthy matter or inorganic elements and other impurities present along with the drug.

Ash values are mainly used in judging the purity and quality of the drug is the indicative of contamination, substitution and adulteration. The total ash usually consists of carbonate, phosphate and silicates. Total ash was found to be 9.66±1.53%w/w. The acid insoluble ash indicates contamination with siliceous materials like earth and sand. The value was found to be 7.56±0.75%w/w. The value of water soluble ash was found to be 6.83±0.35%w/w. Sulphated ash is obtained by treatment with dil. sulphuric acid where the oxides are converted to sulphates. The value was found to be 10.95±0.41%w/w.

The alcohol soluble and water soluble extractives were found to be 7.95%w/w and 8.35%w/w respectively. Ether soluble extractive was found to be 4.61%w/w. The alcohol soluble extractive value indicates the presence of considerable amount of polar organic salts present in the plant. These constants would help to identify and to standardize the plant by future researchers.

Loss on drying determines the amount of water and volatile matters present in a sample. The loss on drying was found to be 2.50%w/w. The seed powder showed absence of foaming index and swelling index.

The qualitative analysis of heavy metals and in organic elements were carried out. The heavy metals are present within the limits. The percentage compositions of different inorganic elements were determined. The presence of inorganic elements like calcium, sulphate, borate, silver, aluminums, copper, potassium and chloride was found to be 0.57, 1.48, 0.96, 0.26, 1.68, 1.28, 2.20 and 1.46%w/w respectively.

The detailed pharmacognostical studies on the seeds of *Abelmoschus moschatus* Medic provides information on the standardization parameters and physiochemical parameters which is essential for the identification of raw material and also used to differentiate the plant from its adulterants and substitutes.

PHYTOCHEMICAL STUDIES

The results of Phytochemical studies are as follows

Table 6.Percentage yield of successive extracts of seeds of *Abelmoschus moschatus* Medic.

S.NO	EXTRACT	METHOD OF EXTRACTION	PHYSICAL NATURE	COLOUR	YIELD (%W/W)
1.	Hexane	Continuous Hot percolation method using Soxhlet apparatus	Oily liquid	Yellowish brown	5.2
2.	Ethyl acetate		Semisolid	Brown in colour	4.5
3.	Ethanol		semisolid	Dark brown in colour	5.3

Table 7.Qualitative Phytochemical analysis

S.No	Chemical constituents	Powdered drug	Hexane extract	Ethyl acetate extract	Ethanol extract
1.	Steroids	+	-	+	+
2.	Glycosides	-	-	-	-
3.	Saponins	-	-	-	-
4.	Flavonoids	+	-	+	+
5.	Tannins	-	-	-	-
6.	Phenolic compounds	+	+	+	+
7.	Proteins	+	-	+	-
8.	Alkaloids	-	-	-	-
9	Glycosides	-	-	-	-
10.	Terpenoids	+	-	-	+
11.	Resins	+	-	+	+
12.	Nitrogenous compound	+	-	+	+
13.	Fats and oils	+	+	+	+

Note: + indicates presence, - indicates absence

Table 8.Behavior of drug powder with different chemical reagents

S.NO	TEST	REAGENT	REACTION	RESULT
1.	Alkaloids	Dragendroffs reagent	No orange red ppt	Absence
2.	Carbohydrates	Fehlings I and II	No red colour	Absence
3.	Flavonoids	Lead acetate solution	Intence yellow colour	Presence
4.	Glycosides	Anthrone + Sulphuric acid	No reaction	Absence
5.	Gum	Hcl+ Benedicts test	No reaction	Absence
6.	Resins	Acetone	No reaction	Absence
7.	Phenols	5% ferric chloride	Formation of blakish violet to pinkish red	Presence
8.	Proteins	Picric acid	Violet colour	Presence
9.	Saponins	Shake with water	No frothing	Absence
10.	Steroids	Acetic anhydride + sulphuric acid		Presence
11.	Tannins	Lead acetate test	No reaction	Absence
12.	Terpenoids	Tin + Thionyl chloride	Pink red colour	Presence
13.	Fats and oils	Saponification test		Presence
14.	Mucilage	Rhuthenium test	No reaction	Absence

Note: + indicates presence, - indicates absence

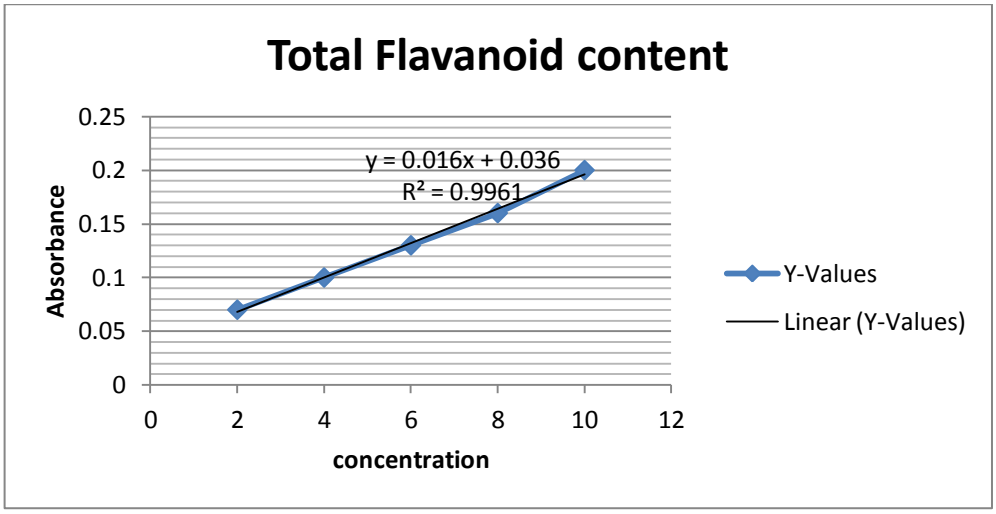
QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

TOTLA FLAVANOID CONTENT

Table 9. Determination of Total Flavanoid content

S.No	Concentration of standard solution(µg/ml)	Absorbance(765nm)
1.	2	0.07
2.	4	0.10
3.	6	0.13
4.	8	0.16
5.	10	0.20
6.	Ethyl Acetate	0.12
7.	Ethanol	0.17

Figure 23.Standard calibration curve for determination of total Flavanoid Content



TOTAL PHENOLIC CONTENT

Table 10.Determination of Total Phenolic Content

S.No	Concentration of standard solution(µg/ml)	Absorbance
1.	20	0.13
2.	40	0.16
3.	60	0.19
4.	80	0.22
5.	100	0.25
6.	Ethyl Acetate	0.15
7.	Ethanol	0.17

Figure 24.Standard Calibration curve for determination of total Phenolic content

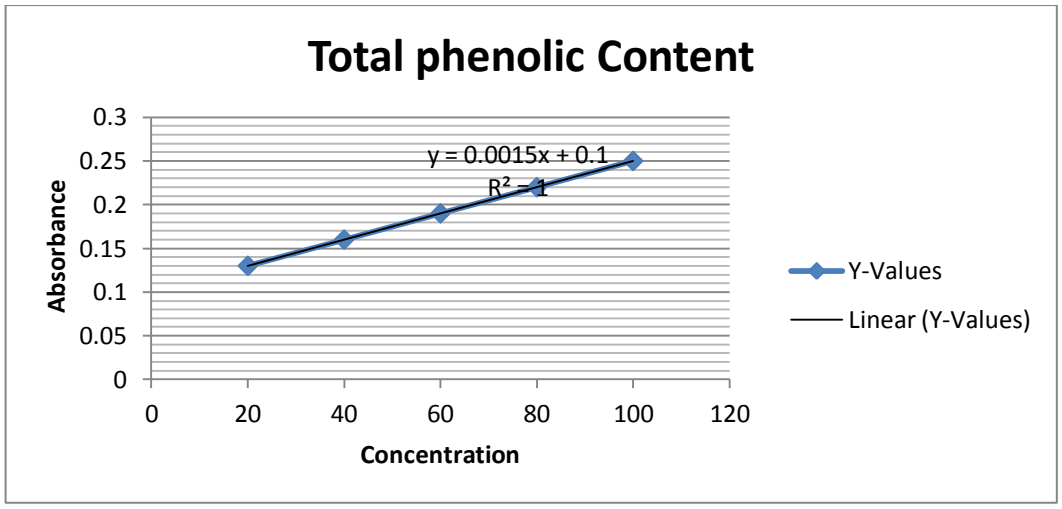


Table 11.Quantitative estimation of phytoconstituents

Total Phenolic and Flavanoid content of extracts of *Abelmoschus moschatus*

S.No	EXTRACT	TOTAL FLAVANOID CONTENT	TOTAL PHENOLIC CONTENT
1.	Ethyl acetate	5.250µg/mg	50.25 µg/mg
2.	Ethanol	8.375µg/mg	70.42 µg/mg

Table 12.Fluorescence characteristic of powdered samples of *Abelmoschus moschatus* at short UV and long UV.

S.NO	TREATMENT	DAY LIGHT	SHORT UV (254nm)	LONG UV (366nm)
1.	Powder	Light brown	Brown	Light black
2.	Powder + water	Light brown	Brown	Blackish brown
3.	Powder + NaoH	Brown	Blackish brown	Brownish black
4.	Powder + Hcl	Dark brown	Blackish brown	Brownish black
5.	Powder + Acetic acid	Brown	Dark brown	Dark brown
6.	Powder + Alc.NaoH	Dull brown	Dark blackish brown	Black
7.	Powder + Picric acid	Light brown	Brown	Dark brown
8.	Powder + Sulphuric acid	Dark brown	Dark brownish black	Dark brown
9.	Powder + Nitric acid	Dark brown	Brownish black	Dark brown
10.	Powder + Iodine	Brown	Pale brown	Black
11.	Powder + $FeCl_3$	Dark brown	Dark brown	Brownish black

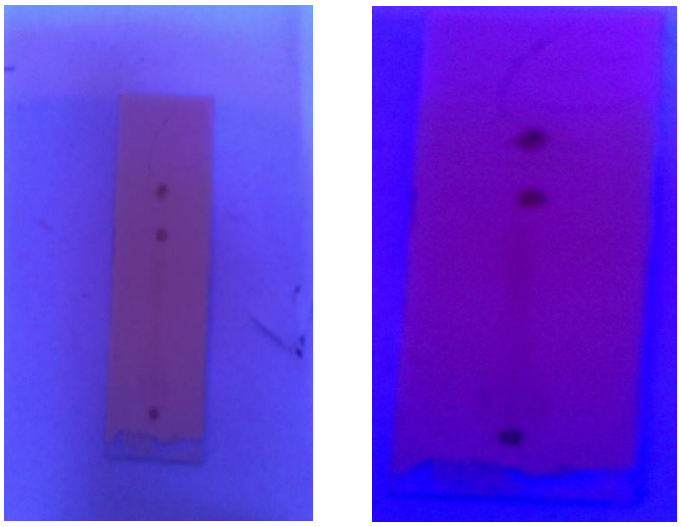
Table 13.Fluorescence analysis of various extracts of *Abelmoschus moschatus* Medic.

S.No	Extracts	Day light	Short UV	Long UV
1.	Hexane	Yellowish brown	Yellowish brown	Brown
2.	Ethyl acetate	Brown	Brown	Dark brown
3.	Ethanol	Brown	Brown	Dark brown

THIN LAYER CHROMATOGRAPHY OF EXTRACTS

Table 14.Thin layer chromatography of Ethyl acetate and Ethanol extract.

S.No	Extracts	Solvent system	No. Of Spots	R_f value
1.	Ethyl Acetate	Ethyl acetate: Formic acid: Glacial Acetic acid: Water(100:11:11:26)	2	0.51 0.69
2.	Ethanol	Ethyl acetate: Formic acid: Glacial Acetic acid: Water(100:11:11:26)	2	0.58 0.68



TLC of ethyl acetate extract

TLC of ethanolic extract

Figure 25, 26. TLC Profile for ethyl acetate and ethanolic extract

HPTLC Finger print Data of Ethanolic Extract of *Abelmoschus moschatus* Medic.

High performance thin layer chromatography (HPTLC) finger printing was performed with the ethanol extract of *Abelmoschus moschatus* Medic. The chromatographic conditions were carried as detailed in material and method of this study. There were 8 peaks observed with different R_f Values and different heights. Percentage of areas was also obtained from the chromatogram.

Table 15.Solvent system

Extract	Solvent System
Ethanol	Ethyl acetate : Hexane (6:4)

Figure 27.TLC Profile for Ethanolic extract

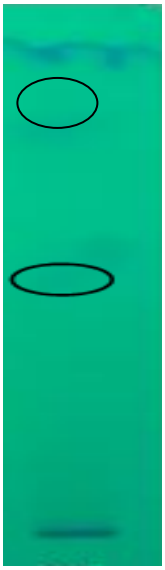
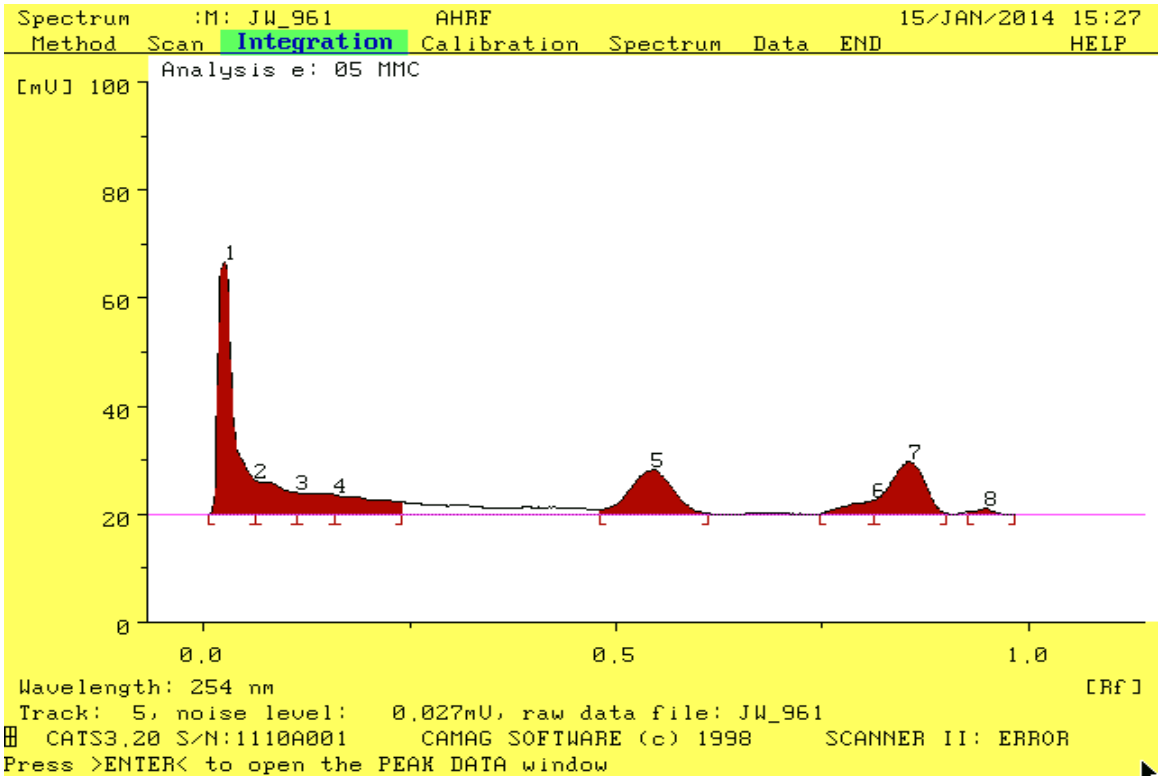


Table 16.HPTLC profile of ethanolic extracts

S.No	R_f	Height	Area	Lambda max
1.	0.02	46.7	678.5	275
2.	0.06	6.3	168.2	290
3.	0.15	4.1	118.2	291
4.	0.16	3.6	155.2	289
5.	0.54	8.2	336.4	282
6.	0.81	2.7	64.9	279
7.	0.85	9.9	317.3	271
8.	0.95	1.1	17.1	280

Figure 28.HPTLC Profile of ethanolic extract



PHYTOCHEMICAL STUDIES

DISCUSSION

Since herbal medicines are prepared from materials of plant origin they are prone to contamination, deterioration and variation in composition. Hence, before proceeding to clinical studies, scientists need a tool to authenticate plants and also to detect their potency.

A lot of analytical techniques have been developed for quality control of drugs from plant origin. Therefore it is very important to undertake phytochemical investigations along with biological screening to understand therapeutic efficacy of medicinal plants and also to develop quality parameters.

In this analysis different polarity of Phyto constituents were sorted out from the coarsely powdered seeds of *Abelmoschus moschatus* Medic., by using solvents of increasing polarity like Hexane, Ethyl acetate and Ethanol by using successive solvent extraction. Successive extractive values revealed the solubility and polarity particulars of the metabolites in the seed powder. Percentage yield of various extracts were as follows, hexane (5.2%w/w), ethyl acetate (4.5gm) and ethanol (5.3gm). Ethanolic extract showed high extractive yield among other extracts.

Qualitative preliminary phytochemical analysis was performed initially with different respective chemical detecting agent to detect the Phyto constituent's nature and their presence in each extract and powder.

Hexane extract showed the presence of steroids, phenols, fats and oils. Ethyl acetate extract showed the presence of steroids, flavanoids, proteins, resins, fats, oils and nitrogenous compounds. The ethanolic extract showed the presence of steroids, flavanoids, phenols, proteins, nitrogenous compound, Terpenoids, resins and fats and oils.

Quantitative estimation of flavanoids, phenols and fluorescence analysis of extracts with different chemical reagents were carried out. Total phenolic content and flavanoid content of ethyl acetate and ethanolic extract were done by calibration curve method and the values were found to be 5.25µg/mg and 8.37µg/mg for flavanoid content and 50.25µg/mg and 70.42 µg/mg for phenolic content respectively.

Qualitative chromatographic analysis of ethyl acetate and ethanolic extracts were done by using Thin Layer Chromatography to separate and identify the single or mixture of constituents present in each extract. The following solvents system was used to separate the phytoconstituents by using Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26).

High performance thin layer chromatography (HPTLC) fingerprinting was performed with the ethanol extract of the seeds of *Abelmoschus moschatus* Medic. There were 8 peaks observed with different R_f values and different heights. Percentage of areas were also obtained from the chromatogram.

SELECTION OF ACTIVE EXTRACT

INVITRO ANTI OXIDANT ACTIVITY

Table 17. Reducing Power Assay

S.No	Concentration	% Inhibition		
		Standard (Ascorbic acid)	Ethanol	Ethyl acetate
1.	10	14.28	13.84	12.24
2.	20	26.84	23.26	21.20
3.	30	48.46	45.25	42.14
4.	40	60.14	56.26	54.15
5.	50	71.86	68.24	65.84

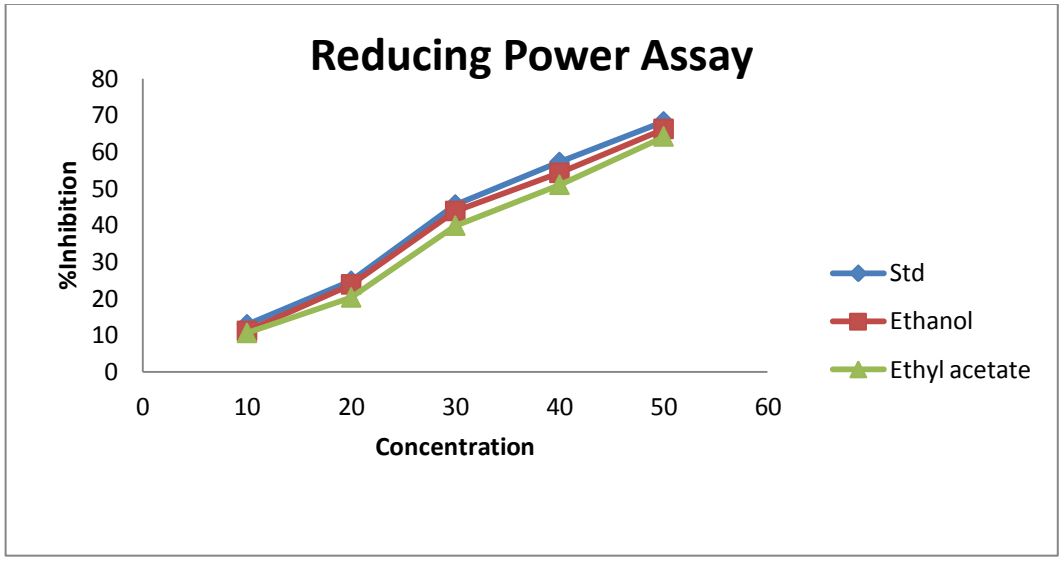


Figure 29.In vitro Reducing power assay

S.NO	GROUPS	IC_{50} VALUE
1.	Standard	33.54
2.	Ethanol	33.60
3.	Ethyl acetate	35.43

HYDROGEN PEROXIDE SCAVENGING ASSAY

Table 18.

S.No	Concentration	% Inhibition		
		Standard (Ascorbic acid)	Ethanol	Ethyl acetate
1.	10	12.95	11.84	10.61
2.	20	24.81	23.46	21.28
3.	30	45.62	44.18	43.59
4.	40	57.21	55.95	53.16
5.	50	68.28	66.21	64.18

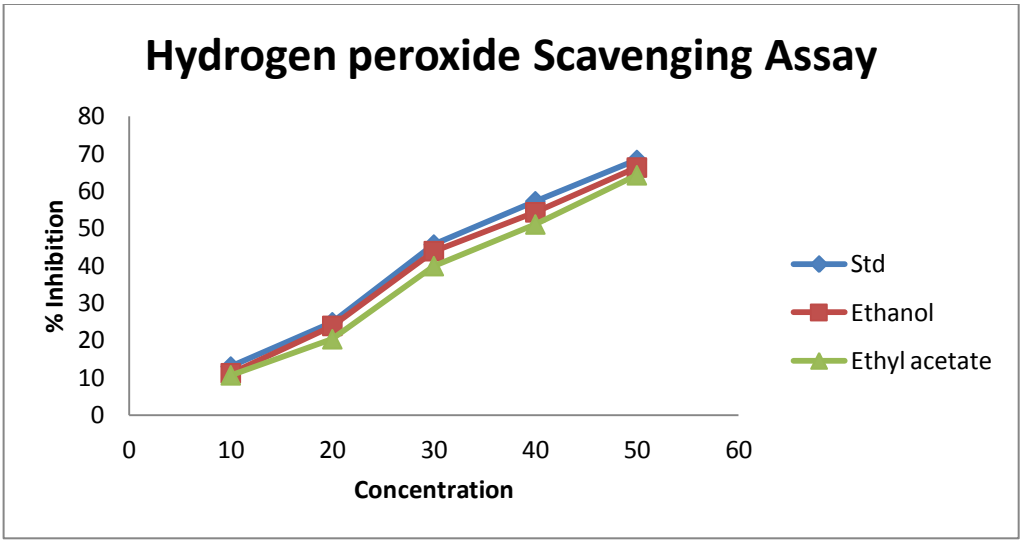


Figure 30.In vitro hydrogen peroxide assay

S.NO	GROUPS	IC_{50} VALUE
1.	Standard	33.54
2.	Ethanol	33.84
3.	Ethyl acetate	35.43

DISCUSSION

Anti oxidant activity and memory enhancing effect are positively correlated. Hence, both ethyl acetate and ethanol extracts are found to posses anti-oxidant activity.

But compared to ethyl acetate, ethanol extract has potent anti oxidant activity as that of standard. So ethanolic extract was selected for further pharmacological studies.

PHARMACOLOGICAL ACTIVITY

ACUTE TOXICITY STUDIES:

The acute toxicity studies were done by OECD guidelines 425. No mortality was observed following the oral administration of seed extracts even with the highest dose 2000mg/kg body weight. So, $1/10^{th}$ and $1/5^{th}$ of this dose (200 and 400mg/kg) were considered as a safety dose for this study.

IN VIVO ACTIVITY PHARMACOLOGICAL STUDIES:

1. **Memory strengthening activity**
2. ***In vivo* antioxidant activity**

MEMORY STRENGTHENING ACTIVITY

- ✓ Effect on Transfer Latency
- ✓ Estimation of brain Acetyl cholinesterase level.

Effect on Transfer Latency (By Elevated Plus Maze):

Transfer Latency (TL) of first day (on seventh day of drug treatment) reflected acquisition or learning behavior of animals. Whereas, TL of next day reflected retention of information or memory. The animals treated orally with 200mg and 400mg/kg showed remarkable reduction in ($P<0.01$) TL of 7^{th} day as well as 8^{th} day, indicating significant improvement in memory. Diazepam (1mg/kg) injected before training significantly increased ($p<0.01$) TL on days 7^{th} and 8^{th} day indicating impairment in learning and memory.

The *Abelmoshcus moschatus* at higher dose level (400mg/kg *p.o* for 7 successive days) successfully reversed memory deficits induced by Diazepam ($p<0.01$), Piracetam (used as the positive control) at a dose of 200mg/kg i.p also improved learning and memory in mice and reversed the memory impairment produced by Diazepam as expected.

Table19. Effect on Transfer latency

S.NO	GROUPS	TL ON 7th DAY (Time in sec)	TL ON 8th DAY (Time in sec)
1.	Control (Vehicle p.o)	46.83± 2.71	30.83±2.04
2.	Disease control	65.17±3.92**	68.50±6.12**
3.	Piracetam control(200mg/kg i.p) + Diazepam	34.83±4.49**	18.00±4.56**
4.	Test drug I(200mg/kg p.o) + Diazepam	45.17±2.85**	39.83±1.60**
5.	Test drug II (400mg/kg p.o) + Diazepam	40.83±3.48**	23.66±3.74*

Values are expressed in mean ± SEM (n=6) ** denotes p<0.01 as compared to control group of young mice, * denotes p<0.05 as compared to control group.(One way ANOVA followed by Dunnett's test)

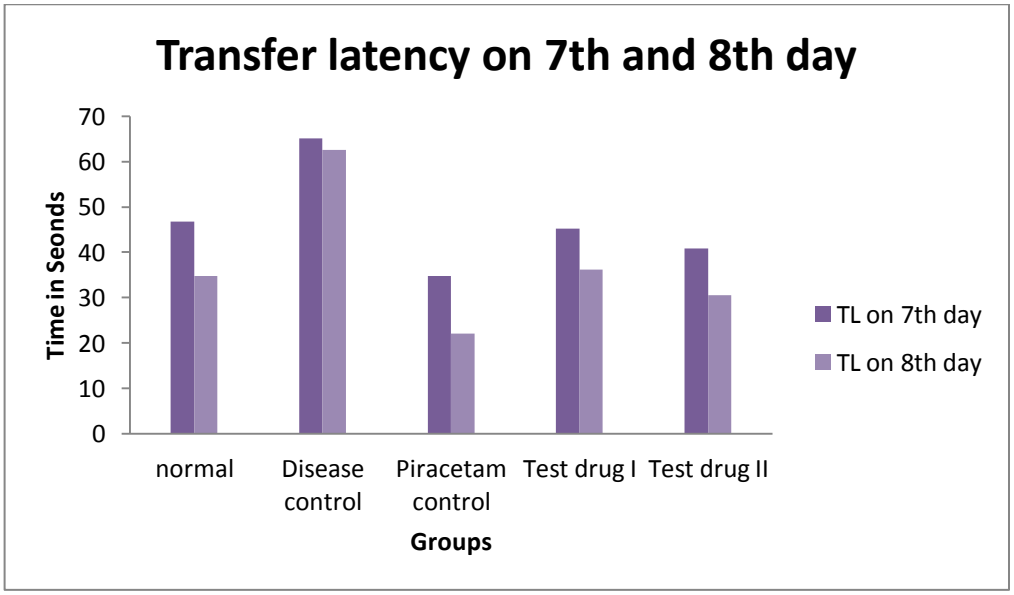


Figure 31.Transfer latency on 7th and 8th day

Estimation of Brain Acetyl cholinesterase level

Abelmoschus moschatus Medic at dose of 200mg and 400mg/kg *p.o* significantly ($p<0.01$) reduced the levels of acetyl cholinesterase as compared to Diazepam treated group by Elman's method, which is considered as indicator of inhibition of Acetyl cholinesterase activity in mice brain after 8 days of treatment. Piracetam (200mg/kg) i.p significantly ($p<0.01$) reduced the levels of Acetyl cholinesterase and indicated in below table.

Treatment Groups	Acetyl cholinesterase level (µmole/min/gm of tissue)
Control (Vehicle p.o)	$3.59 \pm 0.12\times10^{-7}$
Disease control(Diazepam 1mg/kg i.p)	$6.47\pm 0.14\times10^{-7}$**
Piracetam control(200mg/kg i.p) + Diazepam	$2.69\pm 0.09\times10^{-7}$**
AM (200mg/kg p.o) + Diazepam	$4.75\pm 0.06\times10^{-7}$**
AM (400mg/kg p.o) + Diazepam	$3.12\pm 0.02\times10^{-7}$**

Values are expressed as Mean ± SEM, (n=6) and **denotes $p < 0.01$ when compared to control group of young mice. (One way ANOVA followed by Dunnett's test.)

Table 20.Brain AchE level

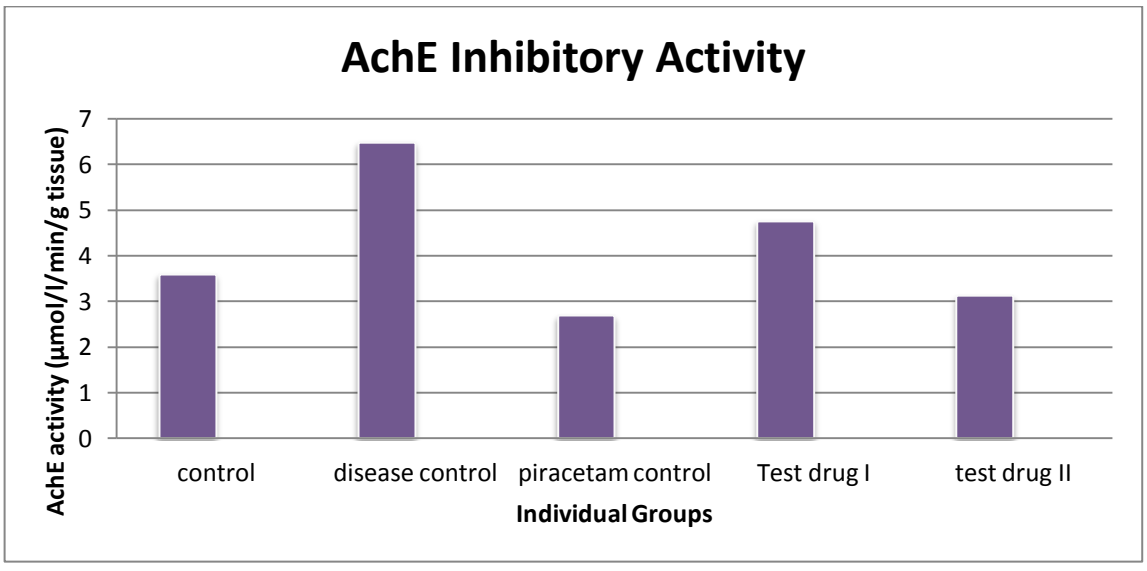


Figure32. AchE inhibitory activity

IN VIVO ANTIOXIDANT ACTIVITY

- ✓ Estimation of Brain Malondialdehyde content.
- ✓ Estimation of Brain reduced Glutathione level.

Estimation of Brain Malondialdehyde content

Abelmoschus moschatus at dose of 200mg/kg and 400mg/kg *p.o* significantly ($p<0.01$) reduced the levels of Malondialdehyde content as compared to Diazepam treated group, which is considered as indicator of inhibition of lipid per oxidase activity in mice brain after 8 days of treatment. Piracetam (200mg/kg) i.p significantly ($p<0.01$) reduced the levels of Malondialdehyde content and indicated in below table.

Treatment Groups	Brain Malondialdehyde content (unit/mg of wet tissue)
Control (Vehicle p.o)	45.55 ± 0.12
Disease control(Diazepam 1mg/kg i.p)	66.57 ± 0.13**
Piracetam control(200mg/kg i.p) + Diazepam	44.46 ± 0.10**
AM (200mg/kg p.o) + Diazepam	50.82 ± 0.05**
AM (400mg/kg p.o) + Diazepam	46.36 ± 0.11**

Values are expressed as Mean ± SEM, (n=6) and **denotes $p < 0.01$ when compared to control group of young mice.(One way ANOVA followed by Dunnett's test.)

Table 21.Effect of *Abelmoschus moschatus* (200mg, 400mg) on brain Malondialdehyde level of mice.

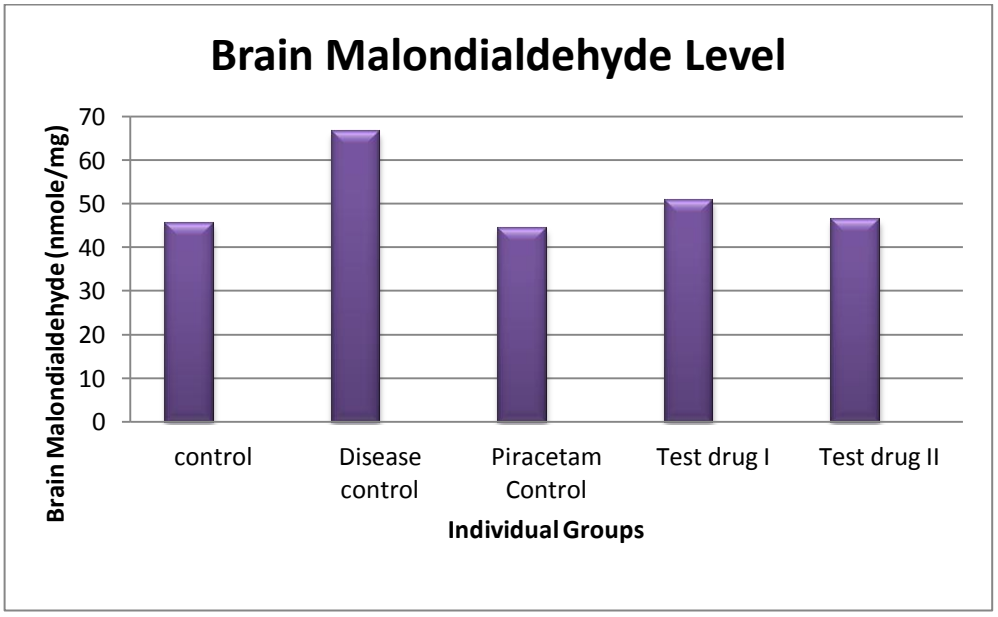


Figure 33.Brain Malondialdehyde level

Estimation of Brain Reduced Glutathione Level

Abelmoschus moschatus showed remarkable increase in brain reduced glutathione level in both Piracetam and *Abelmoschus moschatus* 400mg/kg treated groups. The percent decline in the reduced Glutathione level were 24.88% ($p<0.01$) and 27.47% ($p<0.01$) at AM concentration200, 400mg.

Treatment Groups	**Brain Reduced Glutathione (unit/mg of wet tissue)**
Control (Vehicle p.o)	23.31 ± 0.17
Disease control(Diazepam 1mg/kg i.p)	17.23 ± 0.04**
Piracetam control(200mg/kg i.p) + Diazepam	28.40 ± 0.12**
AM (200mg/kg p.o) + Diazepam	24.87 ± 0.11**
AM (400mg/kg p.o+ Diazepam	27.46 ± 0.21**

Table 22.Brain reduced glutathione level

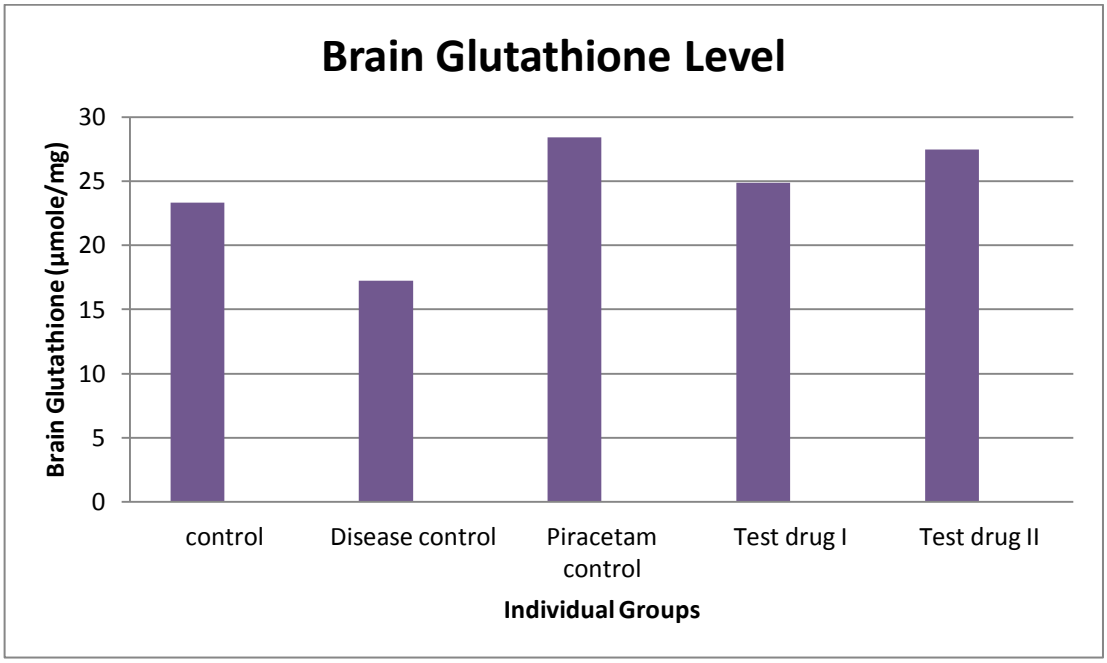


Figure 33.Brain reduced Glutathione Level

DISCUSSION

The present study has been carried out to evaluate the potential of seeds of *Abelmoschus moschatus* Medic. on memory strengthening activity.

The ethanolic extract which showed potent anti oxidant activity was selected for the *in-vivo* study. Based on the acute toxicity studies, $1/10^{th}$ and $1/5^{th}$ (200mg and 400mg) were selected for further *in vivo* studies.

The ethanolic extract of seeds of *Abelmoschus moschatus* Medic. (200 and 400mg) when fed with normal diet for 7days improves the memory of mice reflected by reduced TL (Transfer latency) values as when compared to control group (1). Furthermore the extract administration protected the mice from the development of memory deficits observed after diazepam treatment.

Biochemical estimation of different parameter as mentioned above showed the elevation of acetylcholine level by significant reduction of acetyl cholinesterase activity in brain.

Furthermore plant extract decreased the increase potential of MDA level, an indicator of lipid per oxidation index and increased level of reduced glutathione a potential element of free radical scavenging cycle in the brain as compared to control as well as disease control group. Therefore, it appears that *Abelmoschus moschatus* seeds possesses the memory enhancing capacity in the view of its (i) AchE Inhibitory activity (ii) on the basis of its anti oxidant property a significant decreased in MDA level and sharp increase in anti-oxidant process by increase in reduced glutathione level in mice brain.

Hence from the above results, it is concluded that the ethanolic seed extract of *Abelmoschus moschatus* may be useful in treatment of Alzheimer's disease.

9. SUMMARY AND CONCLUSION

The project entitled "Pharmacognostical, Phytochemical and Memory Strengthening activity of the seeds of *Abelmoschus moschatus* Medic.,(Malvaceae) has been achieved by the following results.

- ✓ The literature survey showed that, only scrappy information was available on this shrub. With this scanty information on this plant consistent expectation of unexplored Phyto chemical profile and pharmacological efficacy under Malvaceae family forms the rationale for the study.
- ✓ The pharmacognostical studies provide a protocol of specific distinguishing features of the plant which is used to differentiate it from its closely related species.
- ✓ Morphological study has provided a characteristic identify of seed which has grayish-brown and blackish in colour, kidney shaped, slightly compressed with shallow depression on both sides, marked with minute parallel ridges and furrows.
- ✓ Anatomical studies revealed the presence of **parenchyma cells, epidermal cells, endosperm containing oil globules and embryo.**
- ✓ Powder analysis showed the presence of **Mucilage cells, parenchymatous cells, sclerenchyma cells, abundant endosperm cells and oil globules.**
- ✓ Varying physio chemical parameters such as ash values, extractive values, loss on drying, crude fibre content, swelling index and foaming index were studied.
- ✓ Presence of **calcium** added up its nutritional value and could be attributed to its memory enhancing activity. There is no fluorescence observed in the particular species.
- ✓ The above mentioned specific characters might offer reliable clues for the correct identification of this seed part in crude as well as fragmentary form and also ensure its differentiation from its substituent's and adulterants.
- ✓ Phytochemical evaluation deals with chemical analysis of the extract used for pharmacological screening. Qualitative preliminary phytochemical analysis was performed to detect the phytoconstituents nature and their presence in powder and its various extracts.
- ✓ Ethyl acetate extract showed the presence of **steroids, flavanoids, proteins, resins, fats and oils and nitrogenous compound.** Whereas the ethanolic extract showed the presence of **steroids, flavanoids, phenols, proteins, nitrogenous compound, terpenoids, resins and fats and oils**.
- ✓ Total phenolic content and total flavanoid content of ethyl acetate and ethanol extract were determined quantitatively.
- ✓ Qualitative chromatographic analysis (TLC) was performed to identify the single or mixture of constituents in the extract.
- ✓ The active extract was selected on the basis of *in vitro* anti oxidant activities. Accordingly the **ethanolic extract** with potential anti oxidant activity was selected fo*r in vivo* study.

- ✓ Based on the acute toxicity studies, $1/10^{th}$ and $1/5^{th}$ (200 and 400 mg/kg) of the maximum tolerated dose (2000 mg/kg B.W) were selected for the *in vivo* studies.
- ✓ The ethanolic extract of the seeds of *Abelmoschus moschatus* Medic. was administered orally for seven days showed a **dose dependent and significant improvement in memory** of young mice and it also successfully reversed the memory deficits induced by diazepam. Furthermore a significant **decreased in cholinergic transmission, lipid per oxidation and increase in brain reduced glutathione level** in mice brain accounts for its multifarious beneficial effects such as memory enhancing property, anti cholinesterase and antioxidant property.

From the above mentioned studies it can be concluded that the pharmacognostical standards generated is useful for the proper identification of plant and also to differentiate it from its closely related species and adulterants with the support of *in vitro* studies and Phytochemical screening, the ethanolic extract was subjected to *in vivo* studies. The ethanol extract with low and high dose showed a significant memory strengthening activity. This may be due to one or more phytoconstituents present in it.

10. REFERENCES

1. Vasudevan M and Parle M. Pharmacological actions of *Thespesia populena* relevant to Alzheimer's disease. *Phytomedicine*, 2006, 13; 677-687.

2. Dhingra D, Parle M and Kulkarni SK. Genetic basis of Alzheimer's disease. *Indian journal of pharmaceutical sciences*, 2005, 67; 409-413.

3. Hebert, LE, Scherr PA, Bienias JL, Bennett DA and Evans DA. Alzheimer disease in the U.S.Population: Prevalence estimates using the 2000 census. *Archives Neurology,* 2003, 60; 1119 -1122.

4. https://www.**alz.org**/national/documents/checklist_10signs.pdf

5. www.alz.org>Alzhimer's disease > know the 10 signs

6. www.webmed.com/brain/**memory-loss**.

7. www.nhs.uk/conditions/**memory-loss.**

8. www.alz.org**brain**tour/**alzheimers_changes**.asp

9. Essman EJ ,"The medicinal uses of herbs", *Fitoterapia,* 1984, 55, 279-289.

10. Bhattacharjee SK, *Hand book of Medicinal Plants*, 5th edition, Pointer publishers, Jaipur, 2008; Pg.No.14.

11. Paulose CS, Chathu F, Reaskhan and Krishna Kumar A. Neuroprotective role of *Bacopa Monneri* extract in epilepsy and effect of glucose supplementation during hypoxia: glutamate receptor gene expression. *Neurochem Research,* 2008, 33(9); 1663-71.

12. Mardar M, Voila H, Wasowski C, Fernandez S. Medina JH and Paladini AC. 6-Methyl apigenin and Hesperidins: New valerian flavanoids with activity on CNS. *Pharmacol Biochem behave,* 2003, 75(3); 537-45.

13. Mukherjee PK, Kumar V and Hought PJ. Screening of Indian medicinal plants for anti cholinesterase inhibitory activity. *Phytotheraphy Research,* 2007, 21(12); 1142-5.

14. Joshi H and Parle M. *Nardostychs Jatamansi* improves learning and memory in mice. *J Med Food* 2006, 9(1); 113-8.

15. Kumar A and Kalonia H. Protective effect of *Withania Somnifera* on behavioral and biochemical alterations in sleep disturbed mice. *Indian J Exp Biol.*, 2007, 45(6); 524-8.

16. Vinutha B, Prashant D, Salma K, Sreeja SL, Pratiti D and Padmaja R. Screening of selected Indian medicinal plants for acetyl cholinesterase inhibitory activity. *J Ethnopharmacology*, 2007, 109(2); 359-63.

17. Yeung YY, Hong S and Corney EJ. A short Enantio selective pathway for the synthesis of the Anti-Influenza neuranidase inhibitor oseltamivir from 1,3-Butadiene and acrylic acid. *Journal of American chemical society*, 2006,128 (19); 6310-11

18. Butler MS, The role of natural product chemistry in drug discovery, *J Nat Pro.*, 2004, 67(12); 2141.

19. Salim AA, Chin YW and Kinghorn AD, *Bioactive molecules and Medicinal plants* 5ed, Springer berlin Heidelberg, 2008. Pg.No.13-14.

20. Anonymous. *The Ayurvedic Pharmacopoeia of India*. Part 1, Volume 7, New Delhi: The controller of Publication, 2011, Pg.No.143.

21. Widodo, Oyen LPA and Nguyen Xuan Dung (Eds): Plant resources of south-East asia, Prosea Foundation, Bogor, Indonesia. 1999,(19), Pg.No.53-56.

22. Ilaria Molfetta, Lucia ceccarini, Mario Macchia, Guido Flamini and Pierluigi Cioni. *Abelmoschus esculentus Moench.* and *Abelmoschus moschatus* Medik. Seeds production and analysis of the volatile compounds, *Food chemistry*, 2013, 141(1); 34-40.

23. Dubey Kumari Priyanka and Datta Animesh. Induced Mutagenesis in *Abelmoschus moschatus* Medik. *International research Journal of Pharmacy*, 2012,3(5); 432-435.

24. Ashish R. Warghat, Nandkishor H. Rampure and Prashanth wagh. *In-vitro* callus induction of *Abelmoschus moschatus* Medik by using different hormone concentration. *International journal of pharmaceutical sciences review and research*, 2011, 10(2); 82-84.

25. Christina AJM, Muthumani P. Phytochemical investigation and Anti Lithiatic activity of *Abelmoschus moschatus* Medik. *Intrenational journal of pharmacy and pharmaceutical sciences*, 2013, 5(1); 108-113.

26. Christina AJM and Muthumani P. Phytochemical investigation and Diuretic activity of *Abelmoschus moschatus* Medik. *Intrenational journal of pharmaceutical and chemical sciences*, 2012, 1(4); 1311-1314.

27. Abishek Kumar Singh, Sanjiv Singh and Chandel HS. Evaluation of hepatoprotective activity of *Abelmoschus moschatus* seed in paracetamol induced hepatotoxicity on rat. *IOSR Journal of Pharmacy*, 2012, 2(5); 43-50.

28. RU Li, Changdong wang, Tao Chen and Peng Chen. Quantitative proteomic analysis of cold-responsive proteins in *Abelmoschus moschatus*, *Journal of Animal and Plant sciences*, 2012, 14(3); 2007-2023.

29. Mir ZGul, Lapakshi M Bhakshu, Farhan Ahmad and Anand K Kondapi. Evaluation of *Abelmoschus moschatus* extracts for antioxidant, free radical scavenging, antimicrobial and antiproliferative activities using *In-vitro* assays, *BMC Complementary and Alternative Medicine*, 2011, 11(6); 1-12.

30. Priti Maheshwari and Anil Kumar. Anti microbial activity of *Abelmoschus moschatus* Leaf extracts, *Association of Biotechnology and Pharmacy*, 2009, 3(3); 1-7.

31. Liu IM, Tzeng IT, Liou SS and Lan TW. Improvement of insulin sensitivity in obese zucker rats by Myricetin extracted from *Abelmoschus moschatus. Planta Med.*,2007, 73(10); 1054-1060.

32. Sahoo D, Jena KS, Rout PK and Rao TR. Two-stage solvent extraction of seeds of *Hibiscus Abelmoschus*: Lipid and FA compositions, *Journal of the American oil Chemists Society*, 2003, 80(3); 209-211.

33. Ramu G, Krishna Mohan G, Jayaveera KN, Suresh N and Chandra Prakash. Evaluation of *Abelmoschus* starch as tablet disintegrant. *Indian Journal of Natural Products and Resources*, 2010, 1(3); 342-347.

34. Pandey R, Kalra A, Katiyar N and Kumar S. Nematicidal activity in flowers of *Abelmoschus moschatus* and aromatic plants. *Indian journal of Nematology*, 2001,31(1); 96-98.

35. Claus P. *Pharmacognosy.* 6th edition, London: Henry Kempton, 1970.Pg.No.15-33.

36. Sass JE. *Elements of Botanical Microtechnique.* Newyork: Mc Graw Hill Book Co, 1940 Pg.no.222.

37. Esaau K. *Plant anatomy.* Newyork, John wiley and sons, 1965. Pg.No-767.

38. Kokate CK. Practical Pharmacognosy, 4th ed. Delhi: MK. Jain for Vallabh Prakasan, 2002. Pg.No.123-124.

39. Evans WC. *Trese and Evans Pharmacognosy*. 15th ed. Rajkamal Electric press, 2005. Pg.No.545-547.

40. Divakar C. *Plant drug evaluation*, 2nd ed. Kerala (Ernaculum): CD Remedies, 2002. Pg.No.49-50.

41. *The Ayurvedic pharmacopoeia of India*, Newdelhi, The controller of publications, 2001. Pg.No.143.

42. Mukarjee PK. *Quality control of herbal drugs.* New Delhi, Business horizons, 2002, Pg.No.188-399.

43. Feder, N O'Brien, T P. *Plant Microtechnique.* Some principles and new methods Amer. *J. Bot,*1968, 55; 123-142.

44. Johansen DA. *Plant microtechnique,* New York, McGraw-Hill book company, Inc.1940, Pg.No.26.

45. Anonymous. *Indian Pharmacopoeia.* New Delhi, The controller of publications, Vol II, 1996; Pg.No. 47-60

46. Harbone JB, Phytochemical methods, London, Pharmaceutical press,1968; Pg.No.49-188.

47. *British Pharmacopoeia*. General Medical Council, London, Pharmaceutical press; 1968; Pg.no.1276-1283.

48. Kokate CK, Purohit AP and Gokahle SB, *Pharmacognosy,* 24th edition. Pune: Vallabh Prakashan, 2003. Pg.No.593-597.

49. Lira, Sergio, Peter Brush, Laurence Senak, Chi San Wu and Edward Malawer. "The use of Inductively coupled plasma-optical emission spectroscopy in the determination of heavy meatls in crospovidone and povidone as a Replacement for the concomitant visual comparison test". *Pharmacopoeial Forum*, (Nov-Dec.2008): vol.34 (6).

50. Archa Vermani, Narneet Prabhat and Avinash Chauhan. Physiochemical analysis of ash of some medicinal plants growing in Uttarkhand. *Nature and Science*, 2010; 8(6); 88-91

51. Harborne JB. Phytochemical methods. *A guide to modern techniques of plant analysis*. 2nd edition. London: Chapman and Hall, 1973. Pg.No.4-34.

52. Tyler VE, Brady LR and Robbers JE. *Pharmacognosy.* 9th edition, Philadelphia USA: Lea and Febrigoree, 1998. Pg.No.78.

53. Khandelwal KR., *Practical Pharmacognosy,* Pune: Nirali prakashan, 1998.Pg.No.20-42.

54. Wallis TE. *Textbook of pharamacognosy.* CBS Publishers and Distributers; Newdelhi, 1985. Pg.No.133-248.

55. Chase CR and Pratt RJ. Fluorescence of powdered vegetable drugs with particular reference to development of a system of identification, *American pharmaceutical system science*,1949, 28;324-333.

56. Kokosi CJ, Kokoski RJ and Slama FT. Fluorescence of powdered vegetable drug under ultraviolet radiation. *Journal of American pharmaceutical association,* 1958, 47(2); 715-717.

57. Edeiga HO, Okwu DE and Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. *African journal of Biotechnology,* 2005,4(7); 685-688.

58. Singleton VL, Orthofer R and Lamuela-Raventos RM. Application and analysis of the folin ciocalteu method for the determination of the total phenolic content from *Limonium brasiliense*, *Molecules,* 2013, 18; 6852-6865.

59. Boham AB and Kocipai AC. Flavonoid and condensed tannins from leaves of *Hawaian Vaccininum vaticulum and viacalycinium. Pacific Science*, 2007, 48; 458-163.

60. Beckett AH, stenlake JB. *Practical Pharmaceutical Chemistry.* 2nd ed. CBS publishers; New Delhi, 2001.Pg.No.115-126.

61. Stahl E. *Thin layer chromatography*. 2nd ed. Newyork Springer-Verlag, 1969. Pg.No.30-160.

62. Wagner H. Plant drug analysis. *A thin layer chromatography Atlas,* 2nded,(Heidenberg); Springer-Verlag Belgium,2002. Pg.No.227-305.

63. Gurdeep R, Chatwal and Sham K Anand. *Instrumental methods of chemical analysis*, Mumbai: Himalaya publishing house, 2007. Pg.No.615.

64. Prashant R.Kaldhone, Yadunath M Joshi, Vilasrao J. Kadam and Prashanth R. Kaldhone studies on *in-vitro* Antioxidant activity of methanolic extract of aerial parts of C*anna indica L*. *Journal of Pharmacy Research,* 2009,2(11); 1712-1715.

65. Saha MR, Hsan SMR. *In vitro* free radical scavenging activity of menthol extract of the leaves of *Mimusops elengi*, *Bang.J.Vet.Med*, 2008, 6(2); 197-202.

66.http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/OECD/OECD- GL423.pdf.OECD Guidelines 423.

67. Itoh J, Nabenshima T, Kameyama T. Utility of an elevated plus maze for the evaluation of nootropics, Scopolamine and electro convulsive shock, *Psychopharmacology,* 1990,101(1);27-33.

68. Dhingra D, Parle M, Kulkarni DK. Memory enhancing activity of *Glycyrrhiza glabra* in mice. *Journal of Ethnopharmacolog,* 2004, 91(2-3); 361-365.

69. Ellman GL, Courtney DK, Andres V and Feathestone RM. A new and rapid colorimetric determination of acetyl cholinesterase activity. *Biochemical Pharmacology,* 1961, 7(1); 88-95.

70. Bhattacharya SK, Upadhyay SN and Jaiswal AK. Effect of Piracetam on electroshock induced amnesia and decrease in brain AchE in rats. *Indian journal of Experimental Biology,* 1993, 31(10); 822-824.

71. Srikumaran BN, Ramkumar K and Raju TR and Shankaranarayanan rao BS. Assay of acetyl cholinesterase activity in the brain. *Brain and Behaviour,* 2004, 34(3); 439-440.

72. Bickford PC, Gould T, Briederick L, Chadman K, Polloch A, Young D, Shukitt-Hale B and Joseph J. Antioxidants-rich diets improve cerebellar physiology and motor learning in aged rats. *Brain research,*2007,886; 211-217.

73. Butterfield DA and Lauderback CM. Lipid peroxidation and protein oxidation in Alzheimer's disease: Potential causes and consequences involving amyloid-beta peptide associated free radical oxidative stress. *Free Radical Biology Medicine,* 2002, 32(1); 1050-1060.